Development of novel methods for tannin quantification in grapes and wine

by

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Declaration

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Summary

Phenolic compounds, and condensed tannins in particular, are of utmost importance in red grapes and wine due to their contribution to the sensory properties and potential health benefits. However, the detailed analysis of these compounds is hampered by their complexity and the lack of reliable quantitative analytical methods. In this study, the analysis of wine tannins using different chromatographic methods was evaluated in order to develop an improved methodology for their accurate characterisation and quantification.

Standard compounds for use in calibration were isolated from cocoa using semi-preparative high performance liquid chromatography or purchased commercially. Calibration curves were constructed and relative response factors based on degree of polymerisation (DP), class of compound and mobile phase composition were determined. Response factors were found to vary as a function of DP and class, indicating the errors associated with quantification as (epi)-catechin equivalents as is often done due to the lack of standards.

Both hydrophilic interaction chromatography (HILIC) and reversed-phase liquid chromatography (RP-LC) methods for tannin analysis were developed. For HILIC, an amide column was used, which provided separation according to DP as well as a separation of isomers within specific elution windows. In RP-LC compounds were separated based on hydrophobicity, resulting in separation of isomers, with compounds of various DPs overlapping. In both separation modes, three detectors were connected in series: a photodiode array ultraviolet (UV) detector, a fluorescence detector (FLD) and a quadrupole-time-of-flight mass spectrometer (Q-TOF-MS). FLD was found to be the most sensitive for procyanidins (PCs), while UV demonstrated the best sensitivity toward gallated PCs. Negative electrospray ionisation (ESI)-Q-TOF-MS proved essential in identifying 161 tannin species based on accurate mass data, and was the most selective of the detectors when using extracted ion chromatograms.

Quantification of tannins in 9 red wine samples and a grape seed extracts indicated that each of the detectors was useful for particular compounds. Co-elution caused overestimation of some compounds by UV and occasionally by FLD as well. Nevertheless, there was good agreement between the HILIC and RPLC methods, as well as between the various detectors in each mode. Quantitative data for the red wine and seed samples were in agreement with those obtained in previous studies. The total number of compounds identified (161) and quantified (74 and 41 in HILIC and RP-LC, respectively), was greater than could previously be obtained. Both methods were shown to be viable options for the analysis of condensed tannins in grape and wine samples. HILIC was found to be more sensitive, and therefore HILIC-UV-FLD-Q-TOF-MS is recommended as the method of choice for detailed quantitative condensed tannin analysis.

Opsomming

Fenoliese verbindings, en veral tanniene, is van kardinale belang in rooi druiwe en wyn as gevolg van hul bydrae tot die sensoriese eienskappe en potensiële gesondheidsvoordele. Die gedetailleerde analise van hierdie verbindings word egter belemmer deur hulle kompleksiteit en die gebrek aan betroubare kwantitatiewe analitiese metodes. In hierdie studie is verskillende chromatografiese metodes geëvalueer om ´n gevorderde metode daar te stel vir meer akkurate karakterisering en kwantifisering van tanniene in wyn.

Standaard verbindings vir die gebruik in kalibrasie is kommersieel verkry of geïsoleer van kakao met die behulp van semi-preparatiewe hoëdruk-vloeistof-chromatografie. Kalibrasie kurwes is ontwikkel en relatiewe respons-faktore, gebaseer op graad van polimerisasie (DP), klas van tannien en mobiele fase samestelling, is vasgestel. Daar is gevind dat respons-faktore wissel met die DP, sowel as klas van tannien teenwoordig, wat dui op foute wat dikwels gemaak word met kwantifisering in (epi)-katesjien ekwivalente as gevolg van´n tekort aan kommersieel beskikbare standaarde.

Beide hidrofiliese interaksie chromatografie (HILIC) en omgekeerde-fase vloeistofchromatografie (RP-LC) metodes vir analise van tanniene is ontwikkel. Vir HILIC is ´n amied kolom gebruik, wat skeiding verskaf volgens DP sowel as isomeriese komposisie binne spesifieke eluerings-gebiede. In RP-LC is verbindings geskei gebaseer op hidrofobisiteit, wat lei tot skeiding van isomere, met verbindings van verskillende DP´s wat soms oorvleuel. In beide skeidings vorms is drie detektors in´n reeks gekoppel: "fotodiode reeks ultraviolet" (UV) detektor, ´n fluoressensie detektor (FLD) en ´n kwadrupool-tyd-van-vlug massaspektrometer (Q-TOF-MS). Daar is gevind dat die FLD die mees sensitief vir prosianidiene (PC´s) is, terwyl UV die beste sensitiwiteit teenoor gallaat PC´s toon. Negatiewe elektrosproei ionisasie (ESI)-Q-TOF-MS was noodsaaklik vir die identifisering van 161 tannien spesies gebaseer op akkurate massa data. Dit was die mees selektiewe van die detektors (wanneer geëkstraheerde ioon chromatogramme gebruik word). Kwantifisering van tanniene in 9 rooiwyn monsters en 'n druiwesaad ekstrak, het aangedui dat elkeen van die detektors nuttig was vir spesifieke verbindings.

As gevolg van onvolledige skeiding, is sommige verbindings se vlakke oorskat deur UV en soms ook FLD deteksie. Nietemin was daar goeie ooreenstemming tussen die HILIC en RP-LC metodes sowel as tussen die detektor gebruik in kombinasie met elke metode. Kwantitatiewe data vir die rooiwyn en saad monsters was in ooreenstemming met dié wat in vorige studies verkry is. Die totale aantal verbindings wat geïdentifiseer is (161) en gekwantifiseer is (74 en 41 in HILIC en RP-LC onderskeidelik) was groter as voorheen verkry. Daar is gevind dat beide metodes aanvaarbare opsies is vir die ontleding van gekondenseerde tanniene in druiwe- en wynmonsters. HILIC het beter sensitiwiteit getoon en daarom word HILIC-UV-FLD-Q-TOF-MS aanbeveel.

This thesis is dedicated to

My family and friends who supported me every step along the way along this journey

Biographical sketch

Elsa Terblanche was born on 23 August 1992 in Pretoria, where she grew up and matriculated from Cornwall Hill College in 2010. She then obtained a BScAgric Oenology (specialised) in 2014 at Stellenbosch University. Elsa went straight on to do her Masters at Stellenbosch University under the supervision of Prof André de Villiers, where she was able to combine her love of wine and chemistry.

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Preface

This thesis is presented as a compilation of four chapters. Chapter 3 is written according to the style of the journal Journal of Chromatography A to which it is/was submitted for publication.

Chapter 1 **General Introduction and project aims**

Chapter 2 **Literature review**
Phenolic compounds: Occurrence in red grapes and wine and analysis.

Chapter 3 **Research results**
A re-evaluation of wine tannin quantification: Comparison of HILIC and RP-LC with UV, fluorescence and high resolution mass spectrometry.

Chapter 4 **General discussion and conclusions**

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Chapter 1

Introduction and project aims

1.1 INTRODUCTION

Wine, by definition, is an alcoholic beverage made by fermenting the juice of grapes. It is probably one of, if not the oldest alcoholic beverage known to mankind, having been around since the beginning of civilisation some 8,000 years ago (Pellechia, 2006). Since the discovery and deliberate production of wine, countless advances have been made in an attempt to improve the quality of the product.

The wine industry forms an integral part of the South African, and particularly Western Cape's economy and lifestyle. South Africa is the 8th largest producer of wine in the world by volume, producing 4.1% of the world's wine in 2015 (Anonymous, 2016a). Of the 98 597 hectares of land under vineyards in South Africa, 45.4% comprise red varieties (Anonymous, 2016b).

Phenolic compounds are important constituents of especially red wines, with anthocyanins and condensed tannins being the main phenolic classes. Condensed tannins, which are oligomers and polymers of flavan-3-ols, constitute up to 50% of the total polyphenols in red wines (Kennedy *et al.*, 2006; Arranz *et al.*, 2012). These compounds have received a lot of interest in the last few decades due to the idea sparked by the 'French Paradox' that they may contribute to the health benefits associated with moderate wine consumption (Richard, J.L., Cambien, F. and Ducimetière, 1981; Renaud & De Lorgeril, 1992). Apart from their potential contribution to health benefits, condensed tannins are essential quality contributors in especially red wine as they contribute to the mouthfeel, bitterness and astringency of the wines, as well as playing a role in the colour evolution and ageing potential of wines (Cheynier *et al.*, 2006; Chira *et al.*, 2011).

Despite the immense importance of condensed tannins in wine, relatively little is known about the exact composition of tannin fractions, and therefore reliable quantitative data for wine tannins are still lacking. This provides the incentive behind extensive research focusing on the quantitative and qualitative investigation of wine tannins (Jackson, 2014). However, the extreme chemical diversity of condensed tannins makes their complete characterisation and accurate quantification a major challenge in the fields of analytical chemistry and natural products in particular. To date, no one method has been able to completely separate all of these compounds, let alone characterise and quantify them (Kalili *et al.*, 2013; Lin *et al.*, 2014).

Wine tannins are complex molecules, comprised of oligomers of flavan-3-ols and galloylated derivatives of these oligomers. These condensed tannins may be classified in three groups based on their chemical properties: procyanidins (oligomers of flavan-3-ol units), prodelphinidins (oligomers of trihydroxylated

flavan-3-ol derivatives) and gallated procyanidins (oligomeric flavan-3-ols esterified to gallic acid). Each monomeric unit, irrespective of class, has two chiral centres. Therefore, as the degree of polymerisation (DP) increases, the number of isomers increases exponentially. This complexity has made the analyses of condensed tannins very challenging.

Several methods have been tested and used for the analysis of condensed tannins in grapes and wines, including bulk methods using ultraviolet-visible (UV-Vis) spectrophotometry (Mercurio & Smith, 2008; Aleixandre-Tudo *et al.*, 2015), colorimetric methods (Somers *et al.*, 1977; Somers & Ziemelis, 1985), high performance liquid chromatography (HPLC), nuclear magnetic resonance spectroscopy (NMR) (Géan *et al.*, 2016) and liquid chromatography hyphenated to mass spectrometry (LC-MS) (Kalili & de Villiers, 2009; Delgado De La Torre *et al.*, 2013; Kalili *et al.*, 2013). Each of these methods has limitations. Bulk methods give information about the total tannin content of the sample; however no information regarding the tannin classes are obtained. Colorimetric methods, which involve measurement of UV absorbance at 280 nm and 520 nm for wine and grape samples, may suffer interference from compounds other than the target compounds. NMR is used for structural elucidation of compounds, or qualitative analysis, however does not give quantitative data. Many developments have made HPLC and LC-MS the preferred techniques for tannin analysis (De Villiers *et al.*, 2016). However, the main limitations of chromatographic methods are the lack of standards of higher molecular weight proanthocyanidins, with tannins consequently being quantified as (epi)catechin equivalents (Lazarus *et al.*, 2001; Herderich & Smith, 2005; Kelm *et al.*, 2005).

1.2 AIMS AND OBJECTIVES

The overall aim of this research was to address limitations previously encountered in the chromatographic analysis of condensed tannins by developing novel high performance liquid chromatography methods to enable accurate identification and quantification of condensed tannins in grape seed as well as red wine samples. To achieve this primary aim, the following objectives had to be met:

i. In view of the lack of commercial standards for high molecular weight procyanidins, standards for calibration were to be isolated from cocoa using semi-preparative high performance liquid chromatography.
ii. Investigating the relative response factors of each of the classes of proanthocyanidins in ultraviolet (UV), fluorescence (FLD) and mass spectrometry (MS) detection as a function of mobile phase composition and degree of polymerisation.
iii. Developing and evaluating both reversed-phase liquid chromatography and hydrophilic interaction chromatography methods in combination with UV, FLD and HR-MS detection

for wine tannin analysis. The methods will be compared in terms of separation efficiency, sensitivity and quantitative performance, to establish the best methodology for the characterisation and quantification of condensed tannins in complex matrices such as grape extracts and wine.

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Chapter 2

Literature review

Phenolic compounds: Occurrence in red grapes and wine and analysis

2.1 INTRODUCTION

Fruits and vegetables are an essential part of the human daily diet as they hold many health benefits due to their vitamin, mineral, fibre and phenolic contents (Rajarathnam *et al.*, 2013). Phenolic compounds are characterised by the fact that they have at least one phenolic group (usually more), which are able to reduce oxygen species, organic substrates and minerals (Pérez-Jiménez *et al.*, 2010; Kalili & De Villiers, 2011). They are widely distributed in the plant kingdom and have received much attention in research due to their reported health benefits, such as their antioxidant capacity and importance in reducing the risk of certain cancers and heart diseases (Ruidavets *et al.*, 2000; Muselík *et al.*, 2007; Rajarathnam *et al.*, 2013). Red wine is considered to be one of the most important sources of phenolic compounds in the human diet (Heras-Roger *et al.*, 2016).

Interest in the phenolic composition of wine and their biological effects has been stimulated by the 'French paradox', a term that refers to the protection from cardiovascular disease, in a very broad sense, resulting from the moderate consumption of especially red wine (Renaud & De Lorgeril, 1992; Biagi & Bertelli, 2015). This term first became popular in 1991 when Prof. Serge Renaud referred to it during an interview, though Richard *et al*. had actually coined 'paradoxe française' in 1981 (Richard, J.L., Cambien, F. and Ducimetière, 1981; Bavaresco *et al.*, 2015). The phenomenon first received interest based on the observation that the French population suffered from lower incidences of cardiovascular heart disease (CHD) than the American population, even though both ate similar fatty diets; Prof. Renaud's argument for this was that French people drank wine with almost every meal, regularly and in moderation (Bavaresco *et al.*, 2015). Since then, a significant body of research has focused on the possible health benefits of moderate wine consumption, as well as wine components that contribute to these effects.

Aside from the health benefits ascribed to phenolic compounds, they are also important quality parameters in specifically red wines, as they contribute to the mouthfeel, bitterness and astringency of wine, and also determine the colour intensity and stability as well as chemical stability of wine (Chamkha *et al.*, 2003; Minussi *et al.*, 2003; Clarke & Bakker, 2004; Mercurio *et al.*, 2007; Obreque-Slier *et al.*, 2010b; Kalili & De Villiers, 2011).

Phenolic compounds can be found in the pulp (1%), juice (5%), skins (50%) and seeds (44%) of grapes (Monagas *et al.*, 2005; Mercurio *et al.*, 2007; Kalili *et al.*, 2013; Du Toit & A. Oberholser, 2014) and are extracted into wine during the winemaking process (Monagas *et al.*, 2005). The phenolic content of the resultant wines will be affected by several factors. The key natural factors that determine the phenolic content of wines are the grape variety, vigour of the vine, climatic and geographical factors and berry ripeness at time of harvest (Obreque-Slier *et al.*, 2010b). Winemaking techniques also play a critical role when it comes to the extraction of phenolics from the berries; time of maceration on skins, intensity of

mixing (frequency and duration of pump overs, punch downs and/or thermovinification) of grape must and skins, maturation, fining and bottle maturation may all significantly affect the phenolic content of these compounds in the final product.

As confirmed by sensory evaluation, knowledge of the phenolic content of grapes and wine is critically important, as this would ideally allow winemakers to adapt winemaking practices in order to obtain optimal (not necessarily maximal) phenolic composition in the final product (Monagas *et al.*, 2005 ; Obreque-Slier *et al.*, 2010b).

2.2 WINE PHENOLIC COMPOUNDS: STRUCTURES AND CHEMISTRY

Phenolic compounds are characterised by the presence of a hydroxylated benzene ring (Monagas *et al.*, 2005). According to differences in their aromatic backbone and hydroxylation patterns, phenolics are classified into different groups, primarily flavonoids and non-flavonoids. These groups can then be further subdivided based on their substitution patterns, as illustrated in **Figure 2.1** for the major classes of wine phenolics.

Phenolic compounds are of particular importance in wines due to the health benefits and organoleptic properties alluded to above. Red wines have a far greater concentration of phenolic compounds than white wines, due to the composition of the skins as well as the different winemaking practices applied for red and white grapes. In the following sections, brief overviews of the phenolics of each class found in wine will be presented.

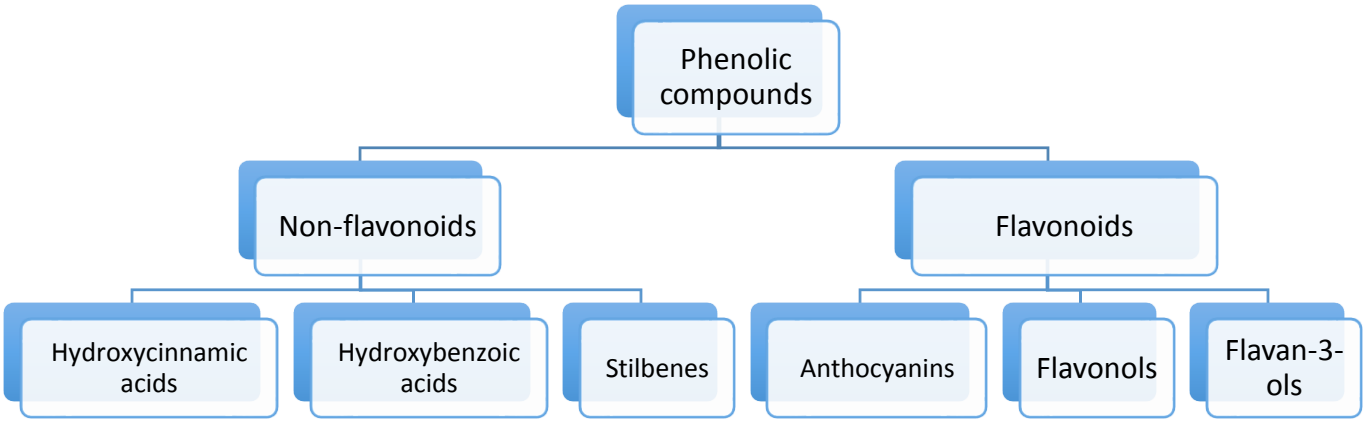


Figure 2.1: Classification of the major classes of grape and wine phenolic compounds.

2.2.1 NON-FLAVONOIDS

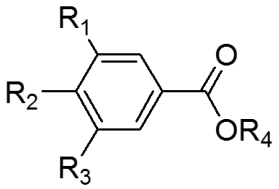
Phenolic acids

Phenolic acids are aromatic secondary metabolites that can be found in a wide variety of fruits and vegetables throughout the plant kingdom, and play a role in the organoleptic as well as quality properties of foods and beverages. The concentrations of phenolic acids are higher in red wines (100-200 mg/L) than in white wines (10-20 mg/L) (Ribéreau-Gayon, Glories, Maujean, & Dubourdieu, 2006). Phenolic acids are phenols possessing a carboxylic acid functionality, and can be further divided into hydroxybenzoic and hydroxycinnamic acids (Minussi *et al.*, 2003; Robbins, 2003).

Hydroxybenzoic acids consist of a C_6-C_1 carbon skeleton and can be found mainly in their glyosidic forms in grapes, whereas in wine the free forms are more prevalent due to hydrolysis of the corresponding esters and glycosides (Ribereau-Gayon *et al.*, 2000; Pérez-Jiménez *et al.*, 2010). The most common hydroxybenzoic acid derivatives present in wine are gallic acid, vanillic acid, syringic acid, protocatechuic acid, gentisic acid, salicylic acid and *p*-hydroxybenzoic acid (Monagas *et al.*, 2005; Ignat *et al.*, 2011), with gallic acid being the most prominent phenolic acid present in grapes (Cheynier *et al.*, 2010).

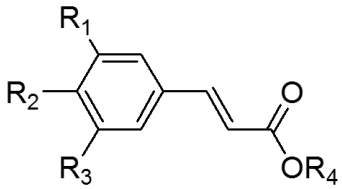
Hydroxycinnamic acids are also phenolic acids, consisting of a C_6-C_3 carbon skeleton (2012). Hydroxycinnamic acids are a major group of phenolics present in grapes, and are the main phenolic compounds present in white wines (Du Toit & A. Oberholser, 2014). The prominent hydroxycinnamic acids in white wines are *p*-coumaric acid, coutaric acid, caftaric acid, fertaric acid, ferulic acid and caffeic acid, which can be found in free or esterified forms (Monagas *et al.*, 2005). The basic structures of the hydroxybenzoic and hydroxycinnamic acids as well as the derivatives commonly present in grapes and wine are presented in **Figure 2.2**.

a.) hydroxybenzoic acids



	R_1	R_2	R_3	R_4
gallic	OH	OH	OH	H
p-hydroxybenzoic	H	OH	H	H
Vanillic	H	OH	OCH_3	H
Protocatechuic	H	OH	OH	H
Syringic	OCH_3	OH	OCH_3	H
Salicylic	H	H	H	OH
Gentisic	OH	H	H	OH

b.) hydroxycinnamic acids

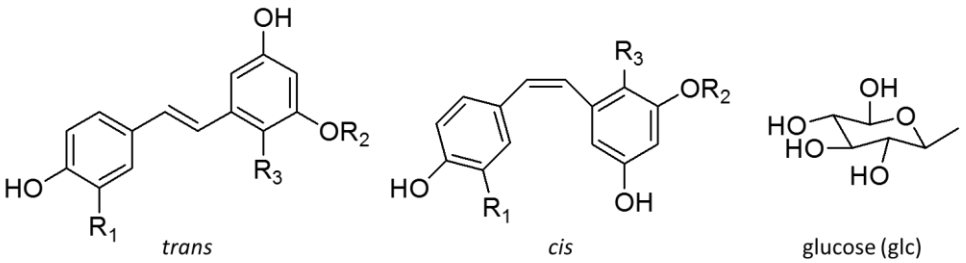


	R_1	R_2	R_3
P-coumaric	H	OH	H
Caffeic	H	OH	OH
Ferulic	H	OH	OCH_3
Sinapic	OCH_3	OH	OCH_3

Figure 2.2: Chemical structures for the phenolic acids commonly found in grapes and wine. R may be H or OH for free acids, or the acid may be esterified.

Stilbenes

Stilbenes are non-flavonoid phenolic compounds comprised of two benzene rings linked by a two-carbon bridge (Ribereau-Gayon *et al.*, 2000). Stilbenes are synthesized by plants in response to ultraviolet (UV) light and fungal infections (Monagas *et al.*, 2005; Fernández-mar *et al.*, 2012). Grapes and products made from them have been found to be the greatest dietary source of stilbenes, with red wine being the richest source of resveratrol (Mattivi *et al.*, 1995; Fernández-mar *et al.*, 2012). These compounds are generally present in the skins of grape berries and thus winemaking practices play a critical role in the extraction of stilbenes; red wines have far greater concentrations of stilbenes compared to white wines due to the skin contact allowed in red wine fermentation, which generally does not take place in white winemaking (Fernández-mar *et al.*, 2012; Vincenzi *et al.*, 2013). The main stilbene of interest is resveratrol, which is present as *cis*-resveratrol in grapes and *trans*-resveratrol in wine. It is mainly extracted into wine during red wine fermentation. Stilbenes, particularly *trans*-resveratrol, have received a lot of attention due to the potential health benefits ascribed to these compounds (Lekli *et al.*, 2010; Guilford & Pezzuto, 2011; Fernández-mar *et al.*, 2012; Kumar & Pandey, 2013; Xiang *et al.*, 2014; Biagi & Bertelli, 2015; Liu *et al.*, 2015; Sancho & Mach, 2015; Silva *et al.*, 2015).



	R_1	R_2	R_3
resveratrol	H	H	H
resveratrol-3-O-glucoside (piceid)	H	glc	H
resveratrol-2-C-glucoside	H	H	glc
astringin	OH	glc	H

Figure 2.3: Chemical structure of important stilbenes in wine.

2.2.2 FLAVONOIDS

All flavonoids, the most abundant of phenolic compounds, share a common structure: 2 aromatic rings (termed A and B, respectively) joined by an oxygenated heterocyclic ring (the C ring) (**Figure 2.4**). Flavonoids can be subdivided into classes according to the functionality/oxidation state of the C ring (Manach *et al.*, 2004; Dai & Mumper, 2010; Kalili & De Villiers, 2011). The flavonoid classes relevant in grapes and wine will be discussed briefly below, with the emphasis on flavan-3-ols, as they are the focus of the research presented.

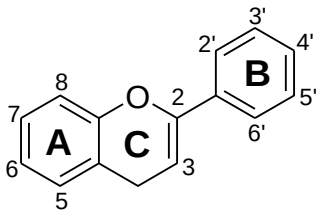
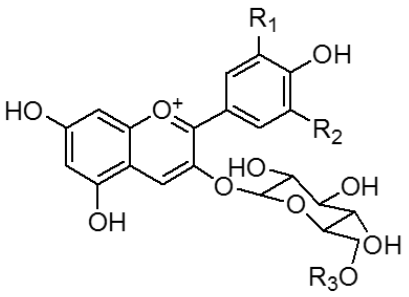


Figure 2.4: The basic flavonoid backbone with carbon numbering indicated**.**

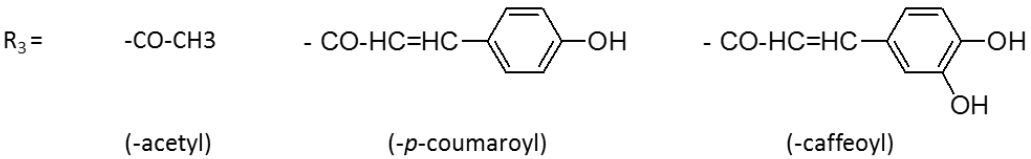
Anthocyanins

Anthocyanins are a very abundant group of flavonoids responsible for the orange, blue, purple and red colours of a variety of fruits and vegetables (Minussi *et al.*, 2003; Manach *et al.*, 2004). An anthocyanin is the glycosylated form of an anthocyanidin. There are six main anthocyanidins, namely cyanidin, delphinidin, petunidin, peonidin, pelargonidin and malvidin present in grapes and wine (**Figure 2.5**), though more than 540 anthocyanin pigments have been found in nature (Monagas *et al.*, 2005; Cheynier *et al.*, 2006; Dai & Mumper, 2010; Willemse *et al.*, 2013). Anthocyanins are found in several fruits and vegetables (though most abundant in fruit), and mainly occur in the skins, with the exception of some fruits with red flesh such as strawberries and cherries (Manach *et al.*, 2004).

Anthocyanins play an important role in red wines, as they impact not only the colour but also the stability and longevity of red wines (Mercurio *et al.*, 2007; Valls *et al.*, 2009). With anthocyanins being found only in the skins of grapes, with the exception of teinturier cultivars, skin contact and mixing of must and skins during maceration and fermentation is essential. In young wines, approximately 200-350 mg/L anthocyanins are present, and the structures become more complex and stable as the wine ages (Clifford & Scalbert, 2000; Es-Safi *et al.*, 2002; Manach *et al.*, 2004). The concentrations of these free anthocyanins decrease as the wine ages, due to reactions that take place with other wine components such as condensed tannins, which form more stable products affecting the wine colour and sensory properties. The reactions that take place include the polymerization of anthocyanins, direct and acetaldehyde-mediated condensation with proanthocyanidins and flavan-3-ols, as well as the formation of pyranoanthocyanins (Fulcrand *et al.*, 1996; Remy *et al.*, 2000; Alcalde-Eon *et al.*, 2004; Vidal *et al.*, 2004; Willemse *et al.*, 2015). The chemical structures of anthocyanins influences their stability; derived pigments are more stable to changes in pH, bleaching by solvents such as SO_2, as well as light and oxidative conditions than grape-derived anthocyanins (Cozzolino *et al.*, 2004; Manach *et al.*, 2004; Mercurio *et al.*, 2007; Valls *et al.*, 2009). Co-pigmentation contributes to the stability of anthocyanins and will be further discussed in the context of flavan-3-ols below.



	R_1	R_2
cyanidin	OH	H
delphinidin	OH	OH
Peonidin	OCH_3	H
Petunidin	OCH_3	OH
Melvidin	OCH_3	OCH_3



(-acetyl) (-*p*-coumaroyl) (-caffeoyl)

Figure 2.5: Chemical structures of the five main anthocyanidins present in grapes and wine.

Flavonols

Flavonols are the most widespread of all the flavonoids present in foods, and are most abundant in onions, kale, broccoli, leeks and blueberries, though they are also found in red wines and tea (Manach *et al.*, 2004). In grapes, these yellow pigments are present in both white and red grape skins (Du Toit & Oberholser, 2014). The most common flavonols in grapes and wine are quercetin, myricetin, isorhamnetin and kaempferol and their derivatives (Manach *et al.*, 2004; Monagas *et al.*, 2005; Castillo-Muñoz *et al.*, 2007, 2009; Flamini *et al.*, 2013; De Rosso *et al.*, 2014; Artero *et al.*, 2015) (**Figure 2.6**). Recent studies have also identified syringetin and laricitrin derivatives in red wines (Hashim *et al.*, 2013; De Rosso *et al.*, 2014). The biosynthesis of flavonols is promoted by light, thus they are generally found in skins of fruit or leaves of plants (Monagas *et al.*, 2005). Flavonols are mostly present in grapes as glycosylated species, with glucose and rhamnose as the most common sugar moieties, and the flavonol profile of wines can be distinguished from that of grapes by the additional presence of aglycone forms as the result of hydrolysis in the acid medium (Manach *et al.*, 2004; Monagas *et al.*, 2005; Du Toit & Oberholser, 2014).

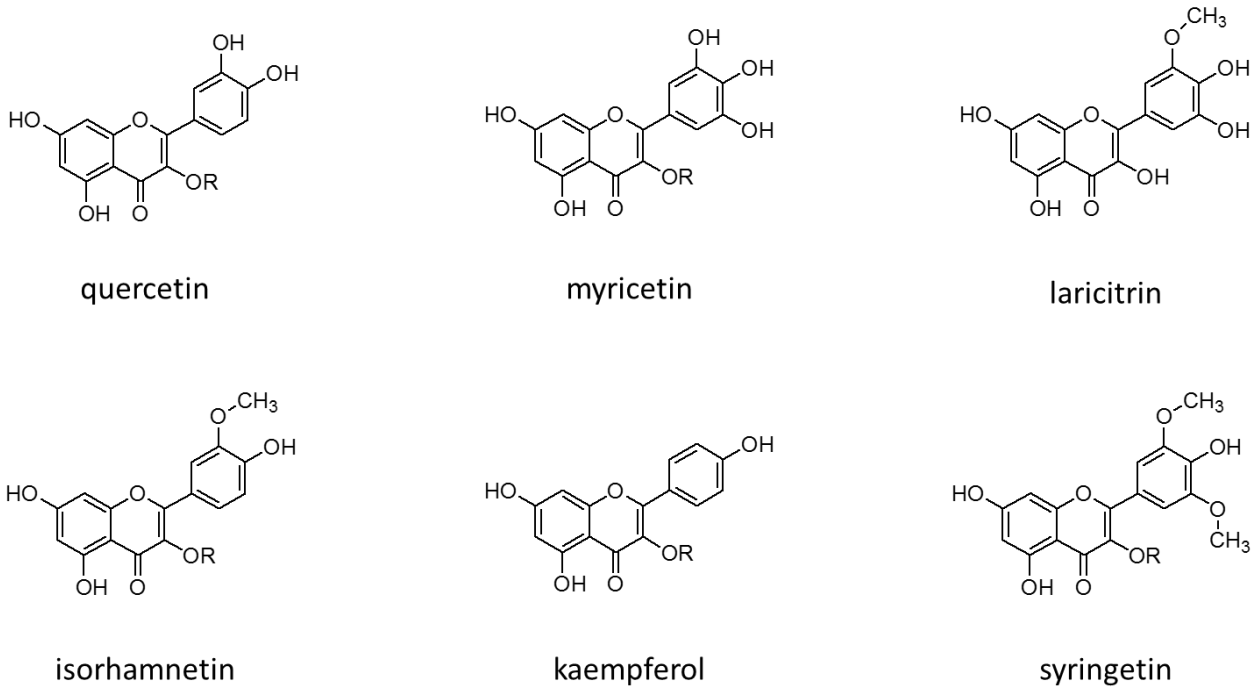


Figure 2.6: Chemical structures for the flavonols most commonly present in grapes and wine. R may be H or a sugar moeity, namely; glucose or galactose.

Flavan-3-ols

Flavan-3-ols are abundant secondary plant metabolites, being the second most widespread natural phenolic compounds after lignin. Apples, green tea and dark chocolate are some of the richest sources of these compounds, but they are also found in grapes and wines (Gu *et al.*, 2004; Manach *et al.*, 2004). These compounds are formed via the shikimate pathway early on in berry development and the quantity does not

change much from *veraison* onwards, though their concentration decreases due to an increased berry size (Du Toit & A. Oberholser, 2014). Flavan-3-ols are found in monomeric forms in foods, as well as in oligomeric (3-10 subunits) and polymeric forms (>10 subunits), referred to as proanthocyanins. Proanthocyanins are divided into several classes based on the substitution patterns of the monomeric flavan-3-ols which they contain. These classes are procyanidins (catechins), prodelphinidins (gallocatechins), propelargonidins (afzelechins), as well as the galloylated derivatives of the first two classes, where the OH at C_3 is esterified with gallic acid (Cheynier *et al.*, 2010) (**Figure 2.7**).

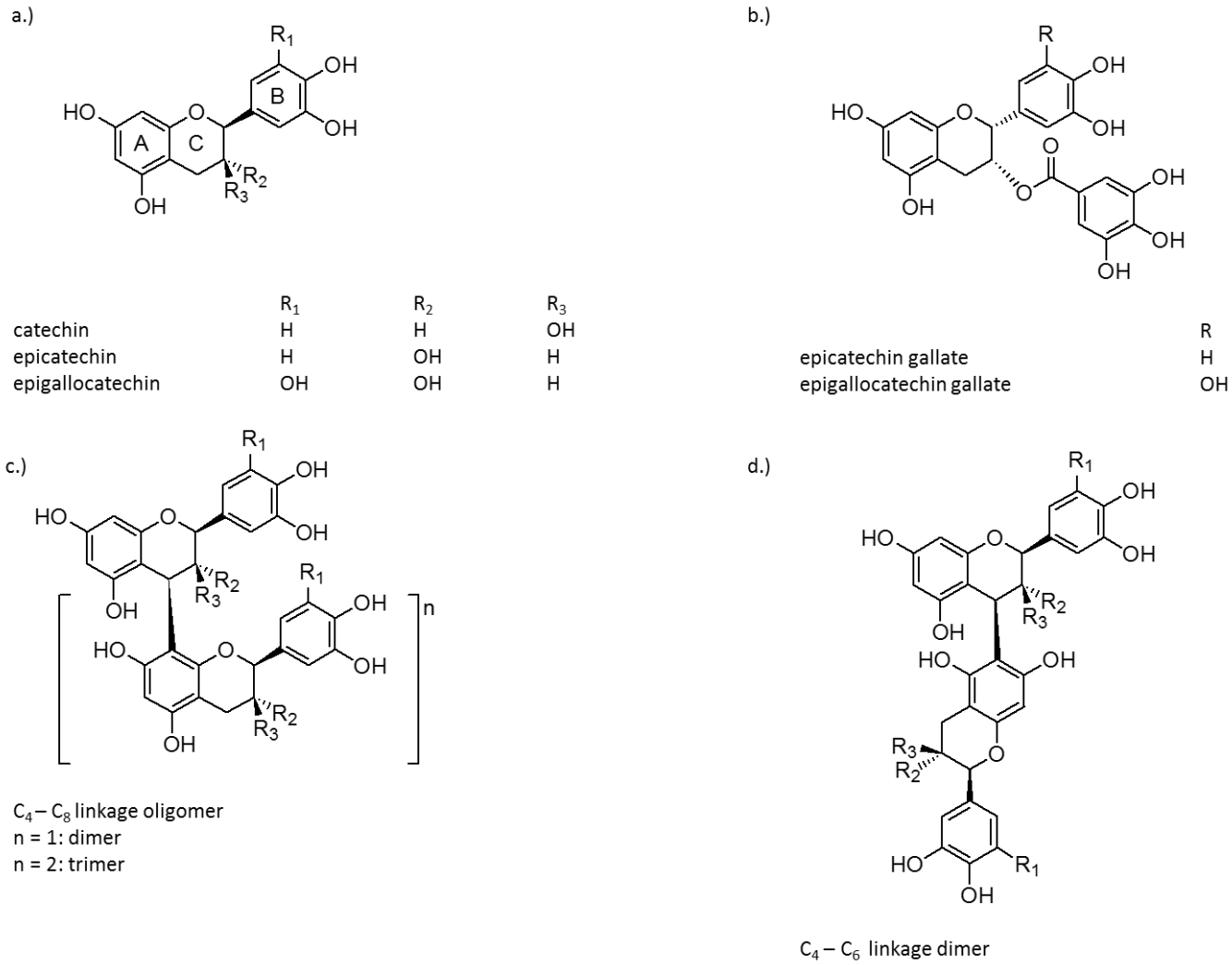


Figure 2.7: Chemical structures for the monomeric and dimeric species of flavan-3-ols found in grapes and wine.

Proanthocyanins, also known as condensed tannins, are formed by interflavan carbon bonds most commonly occurring between positions C_4 and C_8 or C_4 and C_6 (so-called B-type), or less commonly with an additional C_2 - O - C_7 or $C_2 – O – C_5$ bond (A type) (Manach *et al.*, 2004; Cheynier *et al.*, 2010; Dai & Mumper, 2010; Lin *et al.*, 2014) (**Figure 2.7**). Condensed tannins are very important in red wine as they constitute up to 50% of the total polyphenols (Kennedy *et al.*, 2006; Arranz *et al.*, 2012). Flavan-3-ols are chiral compounds, possessing chiral (asymmetric) centres at positions C_2 and C_3 on the C-ring (**Figure 2.7**)

(Schofield, P., Mbugua, D. M., & Pell, 2001). This means that several stereoisomers are possible for monomeric flavan-3-ols. For example, monomeric catechins include 4 possible stereoisomers: (+)-catechin (2R, 3S), (-)-catechin (2S, 3R), (+)-epicatechin (2S, 3S), (-)-epicatechin (2R, 3R). Accordingly, the number of stereoisomers increases exponentially as the degree of polymerisation increases.

Flavan-3-ols are extracted from the skins and seeds of grape berries during maceration. In seeds mainly gallated procyanidins are found and in skins there are procyanidins as well as prodelphinidins, with seeds usually having a greater concentration of tannins than the skins (Vidal *et al.*, 2004; Cheynier *et al.*, 2010; Chira *et al.*, 2015; Allegro *et al.*, 2016). Extraction is increased with skin contact time and the degree of mixing of must and skins. During and after fermentation and pressing, reactions involving proanthocyanins and other phenolic compounds, notably anthocyanins, take place. One of these involves polymerisation through condensation with acetaldehyde, first described in 1976 (Timberlake & Bridle, 1976). In this reaction, the nucleophilic A ring of a procyanidin is substituted with a protonated acetaldehyde molecule. The adduct is then protonated, with the loss of a water molecule forming a carbocation; a second proanthocyanin is then added by nucleophilic attack of the carbocation. The result is a composite proanthocyanin comprising the two units linked through a methylmethine bond (-(CH-CH_3)-), often referred to as an ethyl bridge in oenology. The number of products of this reaction increase with aging of wine, but do not make up a large percentage of the interflavan linkages present. These linkages are also fairly unstable in the acid matrix of wine, thus cleavage of linkages and rearrangements occur frequently (Cheynier *et al.*, 2010). Another reaction involves direct nucleophilic additions of one flavonoid to another. These 'direct' reactions involve nucleophilic addition, where the A ring of one flavonoid acts as nucleophile and the C_4 of another as electrophile. These nucleophilic additions follow several mechanisms and thus yield different products: anthocyanin-proanthocyanin adducts, proanthocyanins-anthocyanin adducts, and anthocyanin polymers and proanthocyanidin polymers etc. (Cheynier *et al.*, 2010). Such pigmented polymers are more stable in the wine matrix than grape-derived anthocyanins and thus contribute to the stability of red wine colour. Due to the complexity of these molecules, including the numbers of compounds and their stereoisomers, their accurate analysis has proved to be one of the greatest challenges in natural products analysis (Mercurio & Smith, 2008).

2.3 TANNINS IN WINE

During wine production, practices are adapted according to the desired style of the wine. With red wine production, grapes are usually crushed and de-stemmed before fermentation and the must is then fermented in contact with the skins, which allows for extraction of phenolics to take place over an extended period of time. In contrast, in white wine production, the must is generally separated from the skins directly after crushing (before alcoholic fermentation) in order to prevent the resultant wine from being too bitter or astringent. For rosé wine production, a limited period of time is allowed on the skins to

extract some colour from the skins of the red grapes. The degree of extraction of phenolics increases with temperature, alcohol and sulphur dioxide concentrations as well as extraction period. The result of these divergent practices is therefore that red wines have much higher phenolic contents, followed by rosé and then white wines (Ribereau-Gayon *et al.*, 2000).

Wine tannins include hydrolysable tannins and condensed tannins, the former derived from oak and the latter from grapes. Oenological tannins, which are commercially available tannin additives derived from mainly oak sources, can also be added to the fermenting must. Hydrolysable tannins are more readily oxidized in wines and thus prevent oxidation of condensed tannins. Since hydrolysable tannins are so reactive, their concentrations in wine are typically very low. In contrast, condensed tannins constitute up to 50% of all phenolics present in red wines (Kennedy *et al.*, 2006; Arranz *et al.*, 2012).

Condensed tannins are very important compounds in specifically red wines because, as mentioned before, they contribute to the health related properties of wine as well as the organoleptic and longevity potential of wines. The sensory properties of tannins have been extensively investigated, particularly with regard to the astringency of red wines (Llaudy *et al.*, 2004; Kennedy *et al.*, 2006). The astringency of a wine is mostly sensorially evaluated, but can also be assessed using the Glories index, which tests the affinity of tannins to bind proteins. This is based on the fact that the sensation of astringency in the mouth is experienced as dryness of the mouth when the tannins bind salivary proteins (Cheynier *et al.*, 2006, 2010).

The astringency of a wine is the sensation of drying and puckering of the mouth as a result of interactions between salivary proteins in the mouth and tannins, and is known to be a positive attribute provided it is balanced with other wine components such as sugar and alcohol (Géan *et al.*, 2016). Bitterness is defined as the sharpness of taste or lack of sweetness and is a result of the taste buds on the tongue's interaction with tannins (Géan *et al.*, 2016), and the perception of bitterness varies with a person's sensitivity to it. The 'harshness' of a wine is the effects of bitterness and astringency combined (Gawel *et al.*, 2000).

Condensed tannins have been shown to affect the bitterness and astringency of particularly red wines (Cheynier *et al.*, 2006; Mercurio & Smith, 2008; Rinaldi *et al.*, 2014; Chira *et al.*, 2015). The reaction between tannins with anthocyanins has been suggested to cause a decrease in the perceived astringency of the wine, and polymerisation and greater degree of galloylation of proanthocyanins appear to increase the astringency (Vidal *et al.*, 2004). Astringency depends on the reaction of protein interaction sites, present on the tannins, with the salivary glands in the mouth. Thus, the bigger the tannin molecule and consequently more protein interaction sites present, the greater number of reactions will occur and the greater the perceived astringency will be.

2.3.1 Hydrolysable tannins

Hydrolysable tannins can occur in fruits, galls, bark, leaves and wood in a variety of plants (Mueller-harvey, 2001). Hydrolysable tannins are classified as gallo- and ellagitannins that release gallic acid and ellagic acid, respectively, upon acid hydrolysis. These compounds are typically esterified around a carbohydrate core, most commonly glucose. The main natural source of hydrolysable tannins in wine is the oak barrels used for ageing, and they are not naturally found in grapes (Ribereau-Gayon *et al.*, 2000; Versari *et al.*, 2013). The main ellagitannins found in oak used for wine maturation are vescalagin and castalagin. The composition of the tannins depends on the species of oak they originate from. European oak species (*Quercus robur*) contain dimeric ellagitannins, whereas American oak species (*Quercus alba*) do not. The different molecules all play a critical role in the ageing of wines aged in oak barrels as they are readily oxidized, and thus prevent oxidation of condensed tannins, while also affecting the flavour properties of the wine (Ribereau-Gayon *et al.*, 2000).

2.4 ANALYSIS OF PHENOLIC COMPOUNDS

Due to the diversity of phenolic compounds and the complexity of many natural products in which they occur, many different methods and techniques have been employed in an attempt to accurately characterise and quantify them. Of these, methods used specifically to analyse proanthocyanidins or condensed tannins will be discussed below.

2.4.1 Bulk methods

Bulk methods, as the name suggests, are used for the analysis of the bulk or total composition of a certain class of compounds within a matrix. In terms of phenolic compounds, bulk methods will be able to quantify the total composition of tannins for example, but not the individual classes or molecular species.

Bulk analysis methods for tannin quantification include precipitation methods and methods based on Ultraviolet-Visible (UV-Vis) spectroscopy. In UV-Vis spectroscopy, absorption of electromagnetic radiation occurs in the range of 200-900 nm wavelengths. UV-Vis spectroscopy is particularly applicable in the wine matrix, because absorbance in this range depends on pi bonds and conjugated double bonds, which are present in phenolic compounds (Aleixandre-Tudo *et al.*, 2015). UV-Vis absorption serves as a means for quantitative analysis, as the amount of radiation absorbed is proportional to the amount (concentration) of compounds. The technique also provides some structural information, as flavonoids display absorption maxima in two UV-Vis ranges and each class displays characteristic absorption spectra. Flavonoids can be distinguished by looking at these two UV-Vis absorption maxima; the first absorption maxima is in the region of 240-285 nm which can be ascribed to the A-ring, and the second maxima is found in the region 300-550 nm and can be ascribed to the B-ring. All flavonoids absorb in the 240-285 nm region and thus the

second absorption maxima gives more useful and selective information; flavan-3-ols, isoflavones, dihydroflavan-3-ols and flavanones only show absorption in the first absorption maxima region, whereas flavonols and flavones absorb light between 300-380 nm in the second absorption maxima range, and anthocyanidins can be easily distinguished from the other flavonoids due to their absorption in the visible range (460-550 nm) (De Villiers *et al.*, 2016). UV-Vis spectroscopy can therefore be used to obtain quantitative information on different classes of flavonoids in wine.

In order to quantify total anthocyanins the Modified Somers Color Assay can be used. The original method has four parts; first wines are analysed in their original state, with UV-spectrum being recorded from 400 – 500 nm, and values at 420 nm and 520 nm being noted. Secondly, excess SO_2 is added so that SO_2-resistant pigments may be measured at 520 nm. Thirdly, the original wine is spiked with excess acetaldehyde, which allows for estimation of the coloured anthocyanins at wine pH by eliminating bleaching from SO_2. Lastly, the wine pH is lowered in order to convert anthocyanins into their coloured forms, where after absorbance is measured at 520 nm and 280 nm, to determine the total red pigments concentration and total phenolics content, respectively (Somers *et al.*, 1977). The modification of this method as suggested by Mercurio *et al.*, is the adjustment of wines to pH 3.4 and the alcohol to 12% v/v prior to any analysis so as to be able to compare results between different samples that originally had varying matrices (Mercurio *et al.*, 2007). For quantification with this method, all absorbance values are converted to 'E', the absorbance value corrected to a 10 mm pathlength. The following calculations can then be used to relatively quantify the various phenolics parameters (Somers *et al.*, 1977):

1. Wine colour density = $E_{420} + E_{520}$
2. Wine colour hue = E_{420}/E_{520}
3. Degree of ionization of anthocyanins (α) = $\frac{E_{520}-E_{520}^{SO_2}}{E_{520}^{HCl}-\frac{5}{3}E_{520}^{SO_2}} \times 100\%$
4. Degree of ionization of anthocyanins after eliminating SO_2 effect = (α) = $\frac{E_{520}^{CH_3CHO}-E_{520}^{SO_2}}{E_{520}^{HCl}-\frac{5}{3}E_{520}^{SO_2}} \times 100\%$
5. Total anthocyanins (mg/L) = $20(E_{520}^{HCl} - \frac{5}{3}.E_{520}^{SO_2})$
6. Ionised anthocyanins (mg/L) = $\frac{\propto}{100} \times (total\ anthocyanins)$
7. Total phenolics (absorbance units) = $E_{280} - 4$
8. Features of 'chemical age'=
   a. $\frac{E_{520}^{SO_2}}{E_{520}^{CH_3CHO}}$
   b. $\frac{E_{520}^{SO_2}}{E_{520}^{HCl}}$

Total phenol quantification can be done very simply in absorbance units by measuring the absorbance of a wine at 280 nm, although this measurement suffers interference from other compounds that also absorb UV light at this wavelength and also doesn't give any information regarding the type of phenolic compounds analysed (Harbertson & Spayd, 2006). The absorbance unit is also an arbitrary unit and therefore doesn't give the most accurate indication of quantity. The Folin-Ciocalteu assay can also be used for determination of total phenolics in wines. This method relies on the fact that phenolic compounds will ionize under alkaline conditions and can then be readily oxidized by the Folin-Ciocalteu reagent to cause a colour change from yellow to blue which can be measured with a spectrophotometer. The problem with this method is that the Folin-Ciocalteu reagent will oxidise unintended compounds in the wine as well, which would lead to the overestimation of phenolic compounds. In order to correct for this, partially, acetaldehyde can be added in order to bind bisulfite, or a correction factor can be used in the case of sweet wines (Harbertson & Spayd, 2006).

Tannin quantification can also be performed using precipitation assays. These methods all rely on selective precipitation of tannins by a suitable reactant, followed by quantification of the precipitated tannins, typically using UV-Vis spectroscopy. Several different reagents have been employed to precipitate tannins out of the matrix: proteins, polymers as well as non-proteinaceous reagents such as polyethylene glycol (PEG), polyvinylpyrrolidone (PVP) and formaldehyde (Aleixandre-Tudo *et al.*, 2015). Of these, the methylcellulose precipitable (MCP) tannin assay and the protein Bovine serum albumin (BSA) tannin assays have been found to show a good correlation between quantitative tannin data and wine astringency (Aleixandre-Tudo *et al.*, 2015), and will be outlined below.

The BSA assay relies on the separation of tannins from the wine matrix by precipitation with the protein bovine serum albumin. The precipitate is then centrifuged to produce a pellet containing the precipitated tannins and proteins, with the supernatant being discarded. This pellet is redissolved in a buffer solution and ferric chloride is added and allowed to react with the solution for ten minutes. A colour reaction takes place between the ferric chloride and phenolic compounds and the absorbance is then measured at 510 nm. A standard calibration curve is set up by measuring the absorbance of the colour reaction between (+)-catechin and ferric chloride and tannin content is expressed in mg catechin equivalents per L (Jensen *et al.*, 2008; Aleixandre-Tudo *et al.*, 2015). The BSA assay was found to have limitations in that beyond a certain concentration of tannins no further precipitation occurs due to the finite amount of BSA and , and where too low concentrations of BSA were present precipitation did not occur, thus tannins can be underestimated in some samples (Jensen *et al.*, 2008). The BSA tannin assay has been widely applied in wines and linked to sensory analyses where it has been found that there is a good correlation between the tannin content quantified using the BSA assay and the perceived astringency of red wines (Mercurio & Smith, 2008; Obreque-Slier *et al.*, 2010a; Ferrer-Gallego *et al.*, 2012; Rinaldi *et al.*, 2014; Harbertson *et al.*, 2015).

The MCP assay is based on the precipitation of tannins by a methylcellulose polymer. This reaction takes place in the presence of ammonium sulphate that then renders the precipitate insoluble and allows it to be separated by centrifugation and measured at 280 nm (Sarneckis *et al.*, 2006; Aleixandre-Tudo *et al.*, 2015). This assay requires for a treatment sample as well as a control, whereby the absorbance of the tannins can be calculated as $A_{control} - A_{treatment}$, and the tannin concentration can then be calculated with a calibration curve in (-)-epicatechin equivalents. The MCP tannin assay has found wide applicability for the quantification of tannins as it precipitates tannins selectively and thus doesn't suffer interference from other phenolic compounds (Sarneckis *et al.*, 2006; Mercurio *et al.*, 2007; Mercurio & Smith, 2008; Aleixandre-Tudo *et al.*, 2015). Aleixandre-Tudo et al. (2015) used the MCP tannin assay to develop a partial-least squares (PLS) model for the quantification of tannins in red wines. A principal component analysis (PCA) was first performed and from this the PLS model was built. Cross-validation was performed within the sample sets, with random selection of calibration and validation sample sets. The model showed promise for the quantification of tannins in South African red wines (Aleixandre-Tudo *et al.*, 2015).

Bulk methods play an important part in the wine industry as they present a simple, robust and high throughput means to quantify total tannins and phenolic compounds in the wines involving minimal analysis time (Mercurio & Smith, 2008). The problems with these methods are however that they lack selectivity (Aleixandre-Tudo *et al.*, 2015), and that assumptions are made regarding the chemical properties of different classes of tannins, which may negatively impact on their accuracy. There is therefore a need for more selective and accurate methods to characterise and quantify tannins, also on the molecular level. This is typically done using high performance liquid chromatography (HPLC) following suitable sample preparation.

2.4.2 Sample preparation

Preparing a sample prior to analysis is often one of the most important steps in the analysis, both from an analytical and economical viewpoint. The choice of sample preparation procedure depends on the sample matrix as well as the analytical method that will be used. Several sample preparation procedures are applicable to phenolic compounds, though for the purpose of this study only those employed in combination with liquid chromatographic analyses will be discussed.

The main objectives of sample preparation are:

1. To remove potential interferents from the matrix, thus increasing the selectivity of the analysis
2. To increase the concentration of the analyte, thus increasing the sensitivity of the method
3. Converting the analyte to a suitable form for detection (if necessary)
4. To provide a robust method that is reproducible regardless of variations in the sample matrix (Smith, 2003).

Sample preparation commences from collection of the fresh sample; target analytes need to be extracted before analysis can take place. For solid samples such as grapes, this is commonly done using solid-liquid extraction (SLE). SLE entails the homogenization of the frozen or dried solid sample, followed by extraction with a suitable solvent. Different solvents are applicable depending on the nature of the analytes; for less polar phenolic compounds extraction is usually performed with relatively apolar solvents such as diethyl ether and/or ethyl acetate. For more polar compounds solvents such as methanol, ethanol or acetone are used (Stalikas, 2007; Hurtado-Fernandez *et al.*, 2010). De-fatting of a sample may also be necessary in some cases - this can be achieved by using dichloromethane or hexane as solvents prior to phenolic extraction. Extraction conditions are determined by the analysis goals and the sample matrix, and usually include shaking or magnetic stirring. Alternative methods such as microwave-assisted, ultrasound-assisted, supercritical fluid extraction and pressurised liquid extraction have also been investigated with the aim of improving extraction time and efficiency. Parameters that have been found to influence the efficacy of the extraction of phenolics are pH, number of extractions and extraction time, temperature, sample weight to solvent ratio and solvent composition (Stalikas, 2007). Extraction of condensed tannins from grape samples has been reported by Kennedy *et al.* using a mixture of acetone and water (2:1) and allowing extraction to take place for 24 hours (Kennedy & Jones, 2001). Mercurio & Smith reported the use of 90% aqueous ethanol for the extraction of Ferco grape seed tannins (Mercurio & Smith, 2008). A study by Bosso *et al.* investigated the use of different solvents for the extraction of seeds and determined that aqueous mixtures of acetone resulted in the greatest extraction of total phenolics and flavonoids (Bosso *et al.*, 2016). Bindon *et al* compared the use of a 'wine-like' extraction using gently crushed grapes, 15% v/v ethanol and 10 g/L tartaric acid, and another extraction using 50% v/v ethanol, pH 2 extraction of a grape berry homogenate. They found that the 'wine-like' extraction showed a better correlation with commercial wines of the same cultivar and thus would be the preferred extraction method should a wine fermentation condition be mimicked (Bindon *et al.*, 2014).

Liquid samples generally require a far simpler preparation procedure, often only requiring centrifugation or filtration before analysis. Dilution or de-alcoholisation may also be required for alcoholic samples. In some cases, pre-concentration or sample clean-up is necessary; for this purpose, solid-phase extraction (SPE), liquid-liquid extraction (LLE) or column chromatography (CC) can be used. For phenolic extracts SPE is the preferred method due to the simplicity, speed, high recoveries and good reproducibility of the technique (Stalikas, 2007). SPE utilizes a disposable cartridge that is prepacked with a stationery phase. A wide range of stationery phases are available, allowing analytes to be trapped based on different mechanisms: polarity, hydrophobicity, size or charged state. Analytes are typically retained on a suitable stationary phase, allowing removal of much of the matrix and therefore a far simpler analysis (Harris, 2010). After trapping the analyte and removing unwanted compounds, the analyte can be released with a small volume of an extraction solvent of suitable polarity or pH (Smith, 2003). Upon retrieval of the analyte, the sample can

then be injected onto the HPLC column as is, or the solvent can be evaporated or diluted. SPE has been widely used for the preparation of wine samples, where HLB (universal polymeric reversed-phase sorbent) and C18 SPE cartridges are often used. Cartridges are typically pre-conditioned with methanol and acidified water prior to sample loading. Water or methanol/water mixtures are used to rinse interferences from the cartridge and target compounds are then eluted with methanol, diethyl ether, ethyl acetate or acetonitrile (Csiktunadi Kiss Forgacs *et al*., 2000; Matějíček *et al.*, 2003; Del Álamo *et al.*, 2004; Pinelo *et al.*, 2006; Jeffery *et al.*, 2008; Perez-Magarino *et al.*, 2008; Manns & Mansfield, 2012; Willemse *et al.*, 2015).

2.4.3 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is an extremely powerful analytical method for the structural elucidation of unknown organic compounds. The technique can be used to study many nuclei, though hydrogen and carbon atoms are most often investigated. NMR gives information about the magnetically distinct atoms in the molecule being studied, and thus information about the environment of the atoms. NMR has proven to be invaluable in the structural elucidation of phenolics (Wolfender *et al.*, 2003; 2010). NMR gives qualitative rather than quantitative information, and coupling HPLC with NMR, which was introduced around 1978, is an incredibly powerful method for the separation and structural elucidation of unknown compounds, even in complex mixtures (Andersen & Markham, 2006). The reason the use of NMR is not as widespread as other analytical methods for the routine analysis of phenolic compounds is due to the complexity and cost of the technique, its limited sensitivity, and the requirement of relatively pure compounds for analysis, which is often hard to achieve for complex samples (De Villiers *et al.*, 2016). NMR has been used to investigate the role of tannins in wines by Géan *et al.*, and they found that the three dimensional structure of tannins affect the reactions with salivary proteins in the mouth and thus perceived astringency, and that tannins have beneficial health properties aside from the suspected antioxidant properties (Géan *et al.*, 2016). Aside from phenolics analysis, NMR has also been used for fingerprinting of wines and identifying varieties from one another (Heintz *et al.*; Son *et al.*, 2009).

2.4.4 High-performance liquid chromatography (HPLC)

In HPLC, high-resolution separation occurs by solvent being forced through finely packed columns at high pressures; analytes are separated by different mechanisms depending on the stationery phase that the column is packed with. The resolution of a separation in HPLC is affected by the column characteristics (particle diameter, length), solid phase, mobile phase and solute characteristics (Harris, 2010). HPLC can be used for qualitative as well as quantitative analysis. Qualitative analysis relies on the characteristic retention times of specific analytes, and spectroscopic detection is often employed in combination with HPLC where qualitative data are required. For quantitative analysis, the peak area or height is utilised as it is proportional to the concentration of the analyte.

HPLC is undoubtedly the preferred method when it comes to phenolic analysis, for analytical as well as preparative purposes (Andersen & Markham, 2006; Valls *et al.*, 2009). The most used columns for phenolic analyses are C18 columns, providing a reversed phase (RP) separation based on hydrophobicity of the compounds. The solvents used typically comprise of an aqueous phase and an organic phase, most often methanol or acetonitrile. The eluent strength increases with an increase of percentage of organic phase. In the case of phenolic analysis, the solvents are also typically acidified with either acetic acid or formic acid (Valls *et al.*, 2009; Fanzone *et al.*, 2010; Delgado De La Torre *et al.*, 2013; Kalili *et al.*, 2013).

Reversed-phase liquid chromatography (RP-LC) is highly efficient for compounds of low molecular weight phenolics, and has been extensively used in the routine analysis of a range of wine phenolics since the first applications in 1978 (Williams *et al.*, 1978; Wulf & Nagel, 1978). The technique is also widely used in the analysis of condensed tannins, where separation of stereoisomers is obtained. The most used columns used are those with C18 stationary phases, though other phases such as C8, C12, phenyl, phenyl-hexyl, pentafluorophenyl, polar embedded RP phases and polymeric RP-LC phases have also been used for flavonoids analysis (Harborne & Boardley, 1984; Kalili & De Villiers, 2011; Manns & Mansfield, 2012; Prokudina *et al.*, 2012; De Villiers *et al.*, 2016). Mobile phases typically consist of aqueous and organic phases with methanol and/or acetonitrile comprising the organic fraction (De Villiers *et al.*, 2016). RP-LC provides a separation based on the polarity of compounds, with more apolar compounds having stronger retention than polar compounds (Santos-Buelga *et al.*, 2003), therefore in the case of proanthocyanidins (PACs) monomers will elute before pentamers, for example. However, for proanthocyanins of a degree of polymerisation higher than 3, the large numbers of isomers mean that complete separation by RP-LC is not possible (Valls *et al.*, 2009), creating the need for other modes of separation for higher molecular weight compounds.

Normal phase (NP-LC) and hydrophilic interaction chromatography (HILIC) provide alternative or complementary information to RP-LC. NP-LC uses a polar stationery phase and a non-polar mobile phase, and polar compounds are therefore highly retained. In the case of proanthocyanidins monomers elute first followed by dimers, trimers etc., while isomers of the same degree of polymerisation co-elute (Natsume *et al.*, 2000; De Villiers *et al.*, 2016). Retention in NP-LC is governed by the adsorption of polar compounds onto the stationary phase, and thus retention increases with an increase in DP. NP-LC has been demonstrated to separate proanthocyanidins efficiently up to DP 10 (Gu *et al.*, 2002; Kelm *et al.*, 2006; Pedan *et al.*, 2015). NP-LC has been applied to South African wines by Alberts *et al.* for the analysis of ethyl carbamate (Alberts *et al.*, 2011), and to grape skin and seed extracts for tannin analysis (Rigaud *et al.*, 1993; Souquet *et al.*, 1996). The use of silica columns gives separation by adsorption chromatography in NP-LC which could lead to lower reproducibility, therefore HILIC is the preferred method between NP-LC and HILIC.

HILIC is an aqueous adaptation of NP-LC, where aqueous acetonitrile mobile phases are used and is especially useful when the molecules being analysed are too polar to be retained on a reversed-phase column (Harris, 2010; Gama *et al.*, 2012). Stationary phases used for HILIC separations are bare silica, diol, polyethylene glycol (PEG), cyclodextrin (CD), ZIC-HILIC and amide (Bernal *et al.*, 2011; Gama *et al.*, 2012; De Villiers *et al.*, 2016). Retention is governed by the partitioning of polar compounds into the aqueous-rich layer on the stationery phase (Gama *et al.*, 2012; De Villiers *et al.*, 2016). HILIC was first applied for the analysis of procyanidins by Lea in 1979 (Lea, 1979), and has increased in popularity ever since. Because of the complementary nature of the retention mechanisms in RP-LC and HILIC, their hyphenation provides a powerful combination for two-dimensional chromatography of proanthocyanidins (Kalili *et al.*, 2013), and this has been investigated in grape and wine analysis in recent years (Kalili *et al.*, 2013; Willemse *et al.*, 2015).

The mode of detection used in combination with HPLC separation also plays an important role in the analysis, as it determines the selectivity and sensitivity of the method. The most used detectors used for phenolics analysis are UV-Vis or photodiode array (PDA), fluorescence (FLD) and mass spectrometry (MS) detectors (Valls *et al.*, 2009; De Villiers *et al.*, 2012). Since all phenolics absorb ultraviolet light, PDA detectors are most commonly used in HPLC, as they allow optimum wavelengths to be chosen for detection of different classes of compounds. Flavonoids have distinctive UV absorption maxima at different wavelengths: flavan-3-ols absorb at 270-290 nm, flavones and flavonols at 270 nm and 330-365 nm, and anthocyanins at 280 nm and 520 nm (Andersen & Markham, 2006; Gómez-Alonso *et al.*, 2007). These characteristic absorption maxima allow for the tentative identification of compound class, as well as determining peak purity. UV-Vis, however, is not very selective for proanthocyanidins due to the fact that all phenolics absorb at 280 nm and not only tannins; therefore if samples analysed are not clean there will be interference from other phenolic compounds and thus the results will be affected. If a pure sample is used, i.e. co-elution does not occur, UV-Vis is a very reliable detector for quantitative means by making use of calibration curves, provided calibration standards are available. UV-Vis detection is non-destructive and can therefore be connected before other detectors such as MS or NMR in a series. UV-Vis is not a very sensitive detector, generally around 0.02-10 ng injected mass is required, depending on the target analyte and the instrumentation (De Villiers *et al.*, 2016). Due to the benefits of structural information UV-Vis provides, the reliability of quantification and the relative low cost of the instrument, it has been widely used for applications in wine and grape samples (Csiktunadi Kiss Forgacs, E., Cserhati, T., Candeias, M.,Vilas-Boas, L., Bronze, R., Spranger, I., 2000; Chamkha *et al.*, 2003; Campo *et al.*, 2007; Castro-López *et al.*, 2014)

FLD detectors provide a more sensitive and selective mode of detection. Compounds that fluoresce are excited by electromagnetic radiation of shorter wavelengths and then emit fluorescent light at longer wavelengths, which can then be detected. The fluorescent light emitted is proportional to the

concentration of the compound and can thus be used for quantitative analysis (Harris, 2010). Quantification in fluorescence is also relatively straightforward, as with UV-Vis, by means of calibration. Most often, calibration is done using catechin or epicatechin and tannins are quantified in the respective equivalents (Waterhouse *et al.*, 2000; Gómez-Alonso *et al.*, 2007; Castro-López *et al.*, 2014). This, however, does not account for the fact that responses of various classes of compounds and degrees of polymerization may vary, and could lead to underestimation of these compounds. The response factor (RF) of a compound refers to ratio of the signal produced by the analyte to the quantity or concentration thereof. A relative response factor (RRF), as may be used for quantitative purposes, is the ratio of the response factors of one analyte to another, for example that of a DP 5 to a DP 1 analyte of the same class (Lin *et al.*, 2012; Lin *et al.*, 2014). The use of FLD detectors however is not as widespread as UV detectors, since very few analytes are able to fluoresce. However, following suitable derivitisation of molecules, this mode of detection can be used to exploit its advantages. Conjugated pi-electrons in aromatic compounds give the most intense fluorescent activity, and thus FLD has proven to be a powerful detection method for particular phenolic compounds that fluoresce in their native state. In wine, these compounds include flavan-3-ols and stilbenes. FLD has been proven to be useful for the detection of flavan-3-ols in wine samples (Gómez-Alonso *et al.*, 2007; De Villiers *et al.*, 2016). Since fluorescence is selective for particularly procyanidins, these compounds have been largely studied with FL detection. A study done by Castro-Lopez *et al.* found that FLD had 3 to 4 orders of magnitude greater sensitivity than a PDA detector depending on the compound analysed (Castro-López *et al.*, 2014). For these reasons, FLD is very useful and beneficial for the analysis of phenolic compounds in grapes and wine.

Phloroglucinolysis

Another way to obtain information on proanthocyanidins is to analyse them after acid-catalysed cleavage. This type of analysis gives information regarding the subunit composition and the interflavonoid linkages (Kennedy & Jones, 2001). These types of analyses are typically used to determine the mean degree of polymerization (mDP) of the proanthocyanidins in a sample. Proanthocyanidins cleave relatively easily under acidic conditions, and thus the method exploits this quality and causes bonds to cleave, forming terminal flavan-3-ol units and electrophilic extension units. These extension units are then trapped by a nucleophile, with benzyl mercaptan and phloroglucinol being the two most used reagents (Kennedy & Jones, 2001). The methods using these reagents are referred to as thiolysis and phloroglucinolysis, respectively. Phloroglucinolysis is stopped by adding aqueous sodium acetate (Kennedy & Jones, 2001), while thiolysis is stopped by cooling the solution (Kennedy *et al.*, 2000). The reaction is followed by HPLC analysis in order to identify and quantify the extension and terminal units. From this information, the ratios of the extension units to terminal units can be compared to determine the mDP (Kennedy & Jones, 2001; Herderich & Smith, 2005). Both thiolysis and phloroglucinolysis have been widely applied for the analysis of

wine procyanidins (Kennedy *et al.*, 2000; Kennedy & Jones, 2001; Bordiga *et al.*, 2011; Hanlin *et al.*, 2011; Downey *et al.*, 2012; Manns & Mansfield, 2012). Phloroglucinolysis is the preferred method in most laboratories since phloroglucinol does not possess the same strong odour as benzyl mercaptan and therefore does not require the same special handling (Kennedy & Jones, 2001). A study by Gu et al. (2003) found red wines to have a mDP 0f 5.2, while a study by Kennedy and Jones (2001) found red grape skins to have a higher mDP (12.2 ± 0.2) than red grape seeds (4.4 ± 0.1).

High performance liquid chromatography-mass spectrometry

HPLC coupled to mass spectrometry (MS) has positioned itself as one of the most important techniques in phenolics analysis, as the separation power of HPLC is combined with the power of MS for identification and/or selective detection. MS allows identification of compounds based on their molecular ions and characteristic fragment ions. One of the main benefits of MS is that only small quantities of a compound are required for analysis (De Pascual-Teresa & Rivas-Gonzalo, 2003). There are several ionization methods available for hyphenation of LC with MS. Those that have found application in the LC-MS of plant secondary metabolites include thermospray (TSP), continuous-flow fast-atom bombardment (CF-FAB), atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). Especially atmospheric pressure (AP)-ESI has made LC-MS more sensitive as well as increased its ease of use (Andersen & Markham, 2006).

ESI is the most widely and almost exclusively used method of ionization for the analysis of phenolic compounds in modern LC-MS (Matrix Assisted Laser Desorption/Ionisation, MALDI, is often used for the direct analysis of solid samples). ESI makes use of electrical energy to transfer ions from solution to gas phase, and this involves three steps; first the dispersal of a fine spray of charged droplets, then solvent evaporation and lastly ion ejection from the highly charged droplets to the mass spectrometer (Ho *et al.*, 2003). ESI can be used for both positive and negative ionization, where positive mode is often used for structural elucidation, though negative mode has been shown to be more sensitive (Rauha *et al.*, 2001). Due to the formed ions with ESI being either monocharged or multiply charged atoms, highly polymerized compounds can also be assigned by using this technique (De Pascual-Teresa & Rivas-Gonzalo, 2003). Owing to the low energy involved in ESI, minimal fragmentation of the ionized molecules occurs, making it invaluable for the detection of molecular ions (De Pascual-Teresa & Rivas-Gonzalo, 2003), although fragmentation is often required for structural elucidation purposes. This can be achieved with collision-induced dissociation (CID) on tandem MS instruments.

The fragmentation behaviour of flavonoids, including proanthocyanins, have been investigated by many researchers (Gu *et al.*, 2002; Li & Deinzer, 2007; De Souza *et al.*, 2008; Hamed *et al.*, 2014). Fragmentation of proanthocyanins involves several pathways, including heterocyclic ring fission (HRF), retro-Diels Alder (RDA) fission, quinone methide (QM) fission and benzofuran-forming (BFF) fission. HRF involves the loss of

the A-ring mainly from the top unit of the oligomer (Gu *et al.*, 2003). With RDA fission, the B-ring is eliminated as a result of the RDA fission of the C-ring (Friedrich *et al.*, 2000; De Villiers *et al.*, 2016); as with HRF, it is primarily the top proanthocyanidin unit that follows this pathway due to it being more energetically favourable. RDA fission is commonly accompanied by a loss of a water molecule. With QM fission, the interflavan bond is cleaved, yielding two monomeric ions in the case of a procyanidin dimer (Friedrich *et al.*, 2000). BFF follows a similar mechanism to HRF and produces a C-ring benzofuran derivative (Li & Deinzer, 2007; De Villiers *et al.*, 2016). The most important fragmentation pathways for proanthocyanins are summarised in **Figure 2.8**.

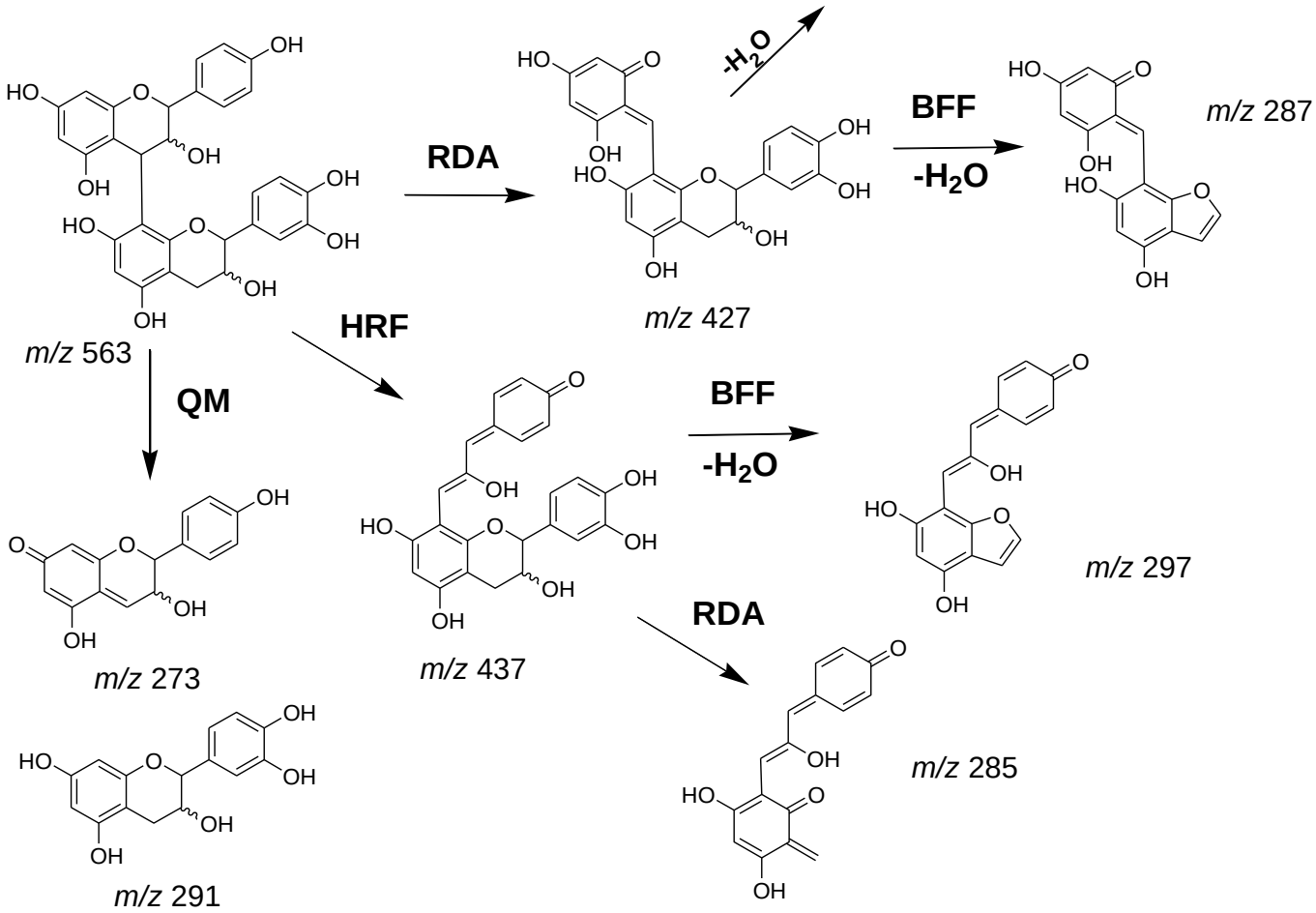


Figure 2.8: General fragmentation patterns of a dimeric procyanidin in positive ionisation ESI-MS. Retro-Diels Alder (RDA), heterocyclic ring fission (HRF), quinone methide (QM) and benzofuran-forming fission (BFF) fragmentation patterns are demonstrated. Source: Reproduced from (De Villiers *et al.*, 2016).

The most commonly used mass analysers in modern LC-MS analysis of flavonoids are time-of-flight (TOF) and quadrupole (Q) systems. A quadrupole mass analyser consists of four metal rods linked to direct current (DC) and radio frequency (RF) voltages, and is used to study the mass to charge (m/z) ratio of ions. A Q-MS system can be used in selective ion monitoring (SIM) or scan mode, where all ions are detected. In

SIM mode, only the specified m/z ratio will have a stable trajectory through the mass analyser and reach the detector whereas all other m/z ratios will not. The advantages of these instruments include low cost and good reproducibility, however they provide low resolution (Brunnée, 1987).

TOF instruments measure, as the name states, the time it takes for ions to move from the ion source to the detector. This time taken to reach the detector is mass-dependent (Brunnée, 1987). TOF instruments are far more expensive than quadrupole instruments, though they provide a high-resolution data, which allows for greater certainty of compound identification with accurate mass information. Tandem MS (MS/MS) systems such a Q-TOF and triple-quadrupole (QqQ) systems are now also being used for structural elucidation and for more selective detection of target analytes. Indeed, LC-MS/MS is an exceptionally powerful tool used for structural elucidation of unknown phenolic compounds, especially when combined with accurate mass information (Passos *et al.*, 2007; Lin *et al.*, 2014; Narduzzi *et al.*, 2015).

While MS is mainly used to structural elucidation and quantification of compounds, it is possible to use it for selective detection and quantification. Availability of calibration standards are essential for quantification in MS, as with the previously mentioned detectors. Quantification can be done with two different approaches; firstly with QqQ in multiple reaction monitoring (MRM) mode for the selective trace-level quantification. MRM allows for detection or precursor ions and 1 or more collision-induced product ions, allowing for deconvolution of overlapping peaks and improving the specificity of the detection (Ong & Mann, 2005; Hammad *et al.*, 2009; Cohen Freue & Borchers, 2012; Lin *et al.*, 2014). MRM has been called the *gold standard* of mass spectrometric quantitation (Ong & Mann, 2005). The second approach is to use high-resolution mass spectrometry, as described by Lin *et al.* where SIM mode was used to select molecular ions of target compounds to be quantified. Relative response factors (to catechin) from UV-Vis detection were translated to MS detection and used for the quantification of molecular ions. The detailed description and formulas for quantification can be found in Lin *et al*. 2014. The disadvantages of this method are that the use of relative response factors based on ion count assumes that there is a constant ionization efficiency for all proanthocyanidins, which needs further evaluation; however a lack of commercially available standards have inhibited this thus far. The SIM peak intensity is also dependent on the solvent ratio at the varying retention times, possible co-eluting compounds and the isomer concentration (Lin *et al.*, 2014) and thus may cause some error in quantification as pertains to MS.

2.5. SUMMARY

Phenolic compounds are of interest due to their proposed health benefits in humans, and therefore the analysis of these compounds is essential. These compounds are also very important quality parameters in especially red wines, where anthocyanins impart the colour and tannins contribute to the mouthfeel,

bitterness and astringency of the wines. Tannins also play a role in the longevity of red wines due to their contribution to chemical stability. Phenolic compounds, and specifically flavonoids, are therefore clearly very important components of especially red wines. Information and research regarding phenolic compounds in South African grapes and wines lags far behind that of competing wine and research groups, thus the need exists for the development of powerful analytical techniques to improve the knowledge of these invaluable compounds and their analysis. Tannins are very important components of wine, as mentioned previously, and thus their accurate characterization and quantitation is important from a research perspective as well as an industry perspective. From an industry perspective having knowledge of tannin content could be invaluable to winemakers and enable them to adapt winemaking practices depending on whether they want to extract more or less of these compounds into their final product. HPLC is the most used method for the qualitative and quantitative analysis of phenolic compounds, however many limitations have been encountered such with regards to their quantitation, and thus it would be of great value to develop a specific method with which to apply the analysis of tannins in grapes and wine. The combination of HPLC with several detection modes has proved to be of great value with regards to structural elucidation and quantification, as neither bulk methods nor single detectors seem ideally suited for this purpose, and thus it is necessary to further assess the possibilities and limitations of existing methods to develop or determine the most suited approach for the accurate characterization and quantitation of tannins in grapes and wine.

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Chapter 3

Research results

A re-evaluation of wine tannin quantification: Comparison of HILIC and RP-LC with UV, fluorescence and high resolution mass spectrometry

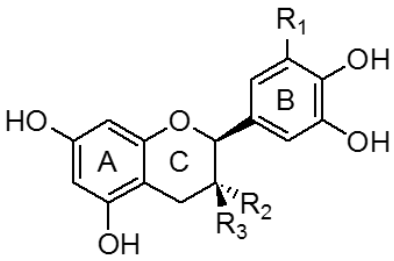
*This chapter is written in the format of J. Chromatogr. A, since the work will be submitted to this journal (Authors: E. Terblanche, W.J. du Toit, A. de Villiers). For this reason, the reference format of the current chapter differs from the rest of the thesis.

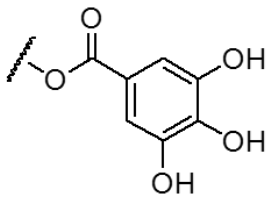
3.1 INTRODUCTION

The two most important and prevalent families of phenolic compounds in wine are proanthocyanidins (PACs) and anthocyanins [1–3]. Proanthocyanidins, or condensed tannins, are of interest not only because of their health benefits [4–10] but also because of their important contribution to the organoleptic properties of wine; these compounds comprise the co-called wine tannins, which contribute to the perceived bitterness and astringency [11–13], and through their interaction with coloured pigments to the colour intensity [14] of red wines in particular.

Condensed tannins are oligomeric flavan-3-ols and their galloylated derivatives. Interflavan linkages most commonly occur between carbons C_4 and C_8 or C_4 and C_6 of the respective flavan-3-ols (the B-type PACs, **Figure 3.1**), or less commonly as C_2-O-C_7 or C_2–O–C_5 A-type bonds (these do not occur in grapes or wine) [1,15–17]. Positions C_3 and C_4 of flavan-3-ol derivatives are chiral centres, thus there are 4 monomeric isomeric structures of catechins: (+)-catechin (2R, 3S), (-)-catechin (2S, 3R), (+)-epicatechin (2S, 3S), (-)-epicatechin (2R, 3R), where (+)-catechin and (-)-epicatechin units are found in *Vitis vinifera* grapes [18]. For the oligomeric or polymeric derivatives of catechins, referred to as procyanidins (PCs), the number of isomeric structures increase exponentially with the degree of polymerisation (DP). PCs are the main phenolic constituents of grape seeds, in addition to their galloylated PC derivatives [19,20]. Furthermore, the corresponding 3',4',5'-tri-hydroxylated flavan-3-ol derivatives (gallocatechins) and oligomeric compounds of this class, prodelphinidins (PDs), are predominantly found in grape skins [21–23].

a.)

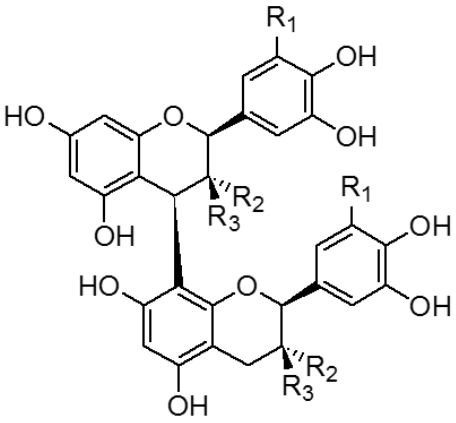


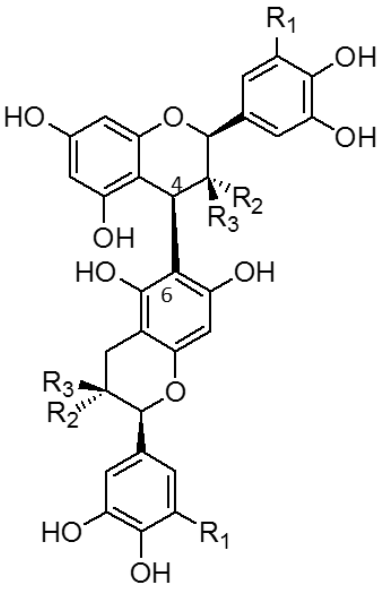


(gallate)

	R_1	R_2	R_3
catechin	H	H	OH
epicatechin	H	OH	H
gallocatechin	OH	H	OH
epigallocatechin	OH	OH	H
catechin gallate	H	H	*gallate*
epicatechin gallate	H	*gallate*	H
gallocatechin gallate	OH	*H*	*gallate*
epigallocatechin gallate	OH	*gallate*	H

b.)





R_1 = H, OH
R_2, R_3 = H, OH, gallate

Figure 3.1: Chemical structures of condensed tannins present in grapes and wine. (A) shows the monomeric structures which are the building blocks of procyanidins, prodelphinidins and gallated proanthocyanidins, and (B) illustrates the principal B-type dimeric proanthocyanins found in grapes and wine.

Condensed tannins are extracted from the grape seeds (for red wine) and skin during maceration and fermentation, and constitute up to 50% of the phenolic content of red wines and are thus very important constituents of the product [24,25]. PACs have been widely studied for their sensory properties in particularly red wines [11,26,27]. The organoleptic properties attributed to tannins are mainly astringency and bitterness. Astringency is the drying and puckering sensation experienced upon drinking wine and is

caused by the interaction between salivary proteins and tannins in the wine [1,26,28]. Bitterness is perceived as the lack of sweetness, or sharpness of taste, and is a result of taste buds' interactions with the tannins; sensitivity toward bitterness varies in humans [28]. These two properties combined are collectively referred to as the 'harshness' of a wine. Although mainly sensorially evaluated, potential astringency can be tested using the BSA tannin assay, which tests the tannins' ability to bind proteins [11–13]. It has been suggested that reactions between anthocyanins and tannins 'soften' a wine leading to a less harsh taste, while higher DP tannins as well as those with a higher degree of galloylation increase the astringency of wines [29–31]. During wine ageing, the PAC content of wine is subjected to several chemical reactions which lead to changes in the tannin composition, and are thought to be responsible for changes in wine mouthfeel [29,30]. Important reactions include acid-catalysed polymerisation or depolymerisation [32,33] and several interactions involving anthocyanins [34–36].

As a consequence of the extreme structural diversity of wine tannins, their complete characterisation, and quantification, remains a significant challenge in oenology in particular and natural product chemistry in general [11,37]. Methods for the analysis of tannins can broadly be grouped into 'bulk' and more detailed molecular methods.

Bulk methods aim to quantify the total tannin composition, irrespective of chemical class or molecular variation. The most common methods used for this purpose are based on UV-Vis spectroscopy, often used in combination with precipitation reactions. It is possible to quantify phenolic compounds by UV-Vis measurement of total phenolics at 280 nm and coloured phenolics at 520 nm, using the Somers or modified Somers Color Assay [38,39]. These methods however suffer interferences from other compounds absorbing at these wavelengths and thus lack specificity. Alternatively, various precipitation methods have been investigated for tannin quantification; these are based on the use of different reactants to precipitate the tannins, followed by the quantification of precipitated phenolics by UV-Vis spectrophotometry [39–43]. Especially the methylcellulose precipitable (MCP) assay, first reported in 1974 [44] and subsequently modified [11,39,40,43], has found relatively widespread application in the wine industry due to its robustness, simplicity and speed.

Although useful for the fast relative quantification of total tannins, the accuracy of bulk methods remains unconfirmed, and these methods provide no information on differences in tannin composition at the molecular level. More detailed and accurate methods for the characterisation and quantification of tannins are available in nuclear magnetic resonance (NMR) spectroscopy [28], high performance liquid chromatography (HPLC) [45–47], mass spectrometry (MS) [48–51] and HPLC coupled to mass spectrometry (LC-MS) [17,52–57]. Of these, HPLC is undoubtedly the preferred method for tannin analysis and quantification [58,59]. Reversed-phase liquid chromatography (RP-LC) is extensively used in the analysis of

low molecular weight (MW) PACs [57,59]. However, incomplete separation of higher MW tannins limits the application of RP-LC for tannin analysis [57,60]. As alternatives, normal phase LC (NP-LC) [53,61,62] and more recently hydrophilic interaction chromatography (HILIC) [63–65] have been used for the separation of PACs according to DP, although information about isomeric composition is lost. Indeed, these modes have found extensive application in the analysis of grape and wine tannins [19,23,66,67].

The most common detectors used in combination with HPLC for tannin analysis include UV-Vis or diode array detectors, fluorescence detectors (FLD) and MS. UV-Vis detection is performed at 280 nm, although the fact that all phenolic compounds absorb at this wavelength [60] places severe demands on chromatographic separation and/or sample preparation for wine tannin analysis. FLD is more sensitive and allows selective detection of PCs, and has therefore found widespread use in the analysis of wine tannins [47], although not necessarily for PDs or gallated PACs [54,60]. Electrospray ionisation (ESI) MS provides a powerful accompaniment to tannin analysis due to the sensitivity, selectivity and structural elucidation characteristics of the technique.

Despite the performance and selectivity of FLD and MS, quantification of individual PACs remains problematic. The lack of commercially available standards, especially for higher DP PACs, implies that often these compounds are quantified as monomeric flavan-3-ol equivalents [54,60,68]. The problem with this has been pointed out by several authors: UV and FLD response factors (RFs, in mass/volume units) vary significantly as a function of DP and chemical class [5,61,69], which would lead to severe underestimation of the levels of higher MW PACs. A further limitation of spectroscopic detectors is that complete chromatographic resolution is essential for accurate quantification; something which is very hard to achieve for complex mixtures of PACs. Although MS is mostly used for structural elucidation, it can also be used for quantitative purposes. Targeted quantification can first of all be done using triple quadrupole (QqQ) instruments in multiple reaction monitoring (MRM) mode [70–72], which is highly selective and sensitive and does reduce the demands on chromatographic separation. However, the lack of standard compounds severely limits this approach for tannin analysis. Alternatively, the inherent selectivity of high-resolution MS (HR-MS) instruments can be used, as has recently been reported for grape seed tannins [17]. For this purpose, the authors derived relative MS response factors (RRFs) for PCs based on simultaneous UV detection and established UV-Vis RRFs for PCs [61]. However, quantification by MS has several drawbacks, firstly in terms of lower linear ranges than other detectors (depending on the instrument used), but also in terms of the formation of multiply charged species and the variation of ion ratios with concentration, and finally due to potential matrix effects when using ESI. For these reasons it is far from clear if HR-MS presents a viable alternative to FLD and UV for the accurate quantification of wine tannins.

The aim of this study was to explore the potential of high performance LC separation in combination with UV, FLD and HR-MS for the quantification of wine tannins. To this end, PCs of various DP, as well as selected gallocatechins and gallated PACs standards were either isolated or purchased commercially to allow investigation of the RRFs for each of these classes of compounds using each of the detectors. Both HILIC and RP-LC methods were developed for PAC separation, and application of the developed methods for the quantitative analysis of tannins in wine and grape seeds were used to critically compare each of the HPLC methods and detectors.

3.2 EXPERIMENTAL

3.2.1 Reagents and materials

(-)-Epicatechin, (-)-epigallocatechin, (-)-epigallocatechin gallate and (-)-epicatechin gallate standards as well as phloroglucinol were purchased from Sigma Aldrich (Steinheim, Germany). Cocoa beans were purchased from a local supermarket. Five commercial red wines of vintages 2015 were obtained from DGB (Pty) Ltd (Wellington, South Africa) and six experimental wines from the Department of Viticulture and Oenology (Stellenbosch University). LC-MS grade acetonitrile and formic acid (99%) were purchased from Merck (Darmstadt, Germany). Deionised water was obtained from a Milli-Q water purification system (Millipore, Milford, MA, USA). Oasis HLB solid phase extraction (SPE) cartridges (6 mL, 500 mg) were purchased from Waters (Milford, MA, USA).

3.2.2 Sample preparation

Cocoa beans

The cocoa beans were prepared as reported by Kalili and de Villiers [73]. Briefly, cocoa beans (2 g) were ground and then de-fatted with (9 mL) hexane. Samples were then extracted first three times with 3 mL 70% acetone in water (v/v) and then three times with 3 mL 70% methanol in water (v/v). The samples were combined and centrifuged. The organic solvent was then removed with a rotovapor and the samples stored under nitrogen until use. The cocoa extract was dissolved in methanol to a stock solution of 1000 ppm prior to chromatographic separation.

Seed samples

The seed samples were prepared using a method slightly adapted from Kennedy and Jones [22]. Seeds were separated from the grape berries and rinsed with deionised water. The seeds were ground fine with a homogeniser and placed in Erlenmeyer flasks with extraction solvent of 2:1 acetone/water in a ratio of 1:10 (sample: extraction solvent), where extraction was allowed to take place for 24 hours at 4°C. After

extraction the samples were frozen overnight and then freeze-dried. The lyophilised samples were reconstituted to a 1000 mg/L stock solution using methanol. The solutions were then sonicated and filtered through 0.45 µm membrane filters prior to injection. For HILIC analyses the samples were diluted (1:1 v/v) with acetonitrile and for RPLC with deionised water.

Wine samples

Condensed tannins were extracted and concentrated from wine samples by means of SPE. The HLB cartridges were pre-conditioned with 2 mL methanol followed by 2 mL deionised water. 10 mL wine was diluted with 40 mL deionised water prior to loading the sample onto the cartridge. The cartridge was then washed with 2 mL water, followed by elution with 8 mL methanol. Recovery was tested by spiking a sample with cocoa, and a recovery of greater than 70% was achieved for all compounds. The effluent was evaporated to dryness using a Rotovapor, reconstituted in 2 mL methanol and later diluted (1:1) with either acetonitrile for HILIC or deionised water for RP-LC analyses.

3.2.3 Instrumentation and chromatographic conditions

Semi-preparative isolation of procyanidin standards

Semi-preparative separations were performed on a modular HPLC system comprising Waters 510 and 501 pumps controlled by a Automated Gradient Controller (Waters) equipped with a Hewlett Packard 1050 variable wavelength detector detector (Agilent Technologies, Waldbronn, Germany) and a manual Rheodyne injector fitted with a 2 mL loop. Detection was performed at 280 nm, and data were recorded using DAx 8.0 data acquisition software (Van Mierlo software, Amsterdam, The Netherlands).

Semi-preparative HILIC separations were performed on a Phenomenex Develosil Diol 100A column (150 × 20.0 mm, 5 µm particles, Torrance, USA). The binary mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B). The gradient used was as follows; 4% - 40% B (0 – 50 min), 40% - 100% B (50 – 55 min), 100% B for 5 min at a flow rate of 7 mL/min. The injection volume was 1.5 mL. PCs of DP 2-5 were collected manually; the fractions of 22 separations were pooled for each DP, and the mobile phase was evaporated using a BÜCHI Rotovapor R-134, frozen and freeze-dried.

Each of the pooled HILIC fractions were reconstituted in methanol and further separated by semi-preparative RP-LC on a Gemini C18 (250 × 10 mm, 10 µm particles) column (Phenomenex). The mobile phases were the same as used for HILIC, with the following gradient: 2% - 18% A (0 – 20.42 min), 18% - 25% A (20.42 – 34.03 min), 25% - 100% A (34.03 – 39.13 min), 100% B until 42.53 min. A flow rate of 5 mL/min was used and 1.5 mL was injected. The major PC isomer for each DP was collected and pooled for 10

injections for the dimer, 3 for the trimer, 2 for the tetramer and 5 for the pentamer. Examples of semi-preparative HILIC and RP-LC separations for the cocoa sample are presented in the Supplementary Information (SI, **Figure S1**).

The collected fractions were evaporated using a Rotovapor, frozen and freeze-dried and stored at -80°C prior to use. The freeze-dried standards were reconstituted at 1000 mg/L in methanol to provide the stock solutions used for calibration purposes.

Analytical HILIC- and RP-LC-UV-FLD-MS analyses

Both HILIC and RP-LC analyses were carried out on a Waters Acquity UPLC system equipped with a binary solvent manager and an autosampler. Three detectors were connected in series: an Acquity PDA detector, Acquity fluorescence detector and a Synapt G2 quadrupole time-of-flight (Q-TOF) mass spectrometer equipped with an ESI source (Waters). The system was controlled using MassLynx v.4.1 software. A column manager maintained the column temperature at 40°C. UV-Vis spectra were recorded between 200 – 500 nm, with quantification performed at 280 nm. Fluorescence detection was performed at two sets of wavelengths: excitation wavelength 230 nm and emission wavelength of 320 nm, and excitation/emission wavelengths of 230/360 nm. Negative ionisation ESI was used, with a capillary voltage of 2.5 kV and a cone voltage of 35 V. The source temperature was 120°C and the desolvation gas temperature 275°C. The cone and desolvation gas flows were 50 and 650 L/h, respectively (both nitrogen). Low collision energy data (6 eV) were obtained in the range of 260 – 2000 amu at a scan time of 0.2 sec. Simultaneous high collision energy data were acquired in MS^E mode using a collision energy ramp of 20-60 eV.

HILIC separations were performed on an XBridge Amide column (150 × 4.6 mm i.d., 2.5 µm particles, Waters) equipped with a Phenomenex KrudKatcher pre-column filter. The binary mobile phase consisted of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid (B). The following gradient was used: 5 – 40% B (0 – 60 min), 40 – 70% B (60 – 62 min), 70% for 2 min, followed by re-equilibration for 8 min. A flow rate of 1 mL/min was used and the flow was split between the FLD and Q-TOF-MS, with 0.3 mL/min entering the MS. RP-LC separations were performed on a Kinetex C18 column (150 × 2.1 mm i.d., 2 µm particles, Phenomenex) fitted with a Phenomenex KrudKatcher pre-column filter. The mobile phases comprised 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B). The following gradient was used: 2 - 30% B (0 – 41.68 min), 30 - 100% B (41.68 – 42 min), 100% B for 4 min before returning to initial conditions for a 4.5 min re-equilibration step. A flow rate of 0.3 mL/min was used and introduced directly into the MS without splitting.

Calibration procedures

Stock standard solutions were prepared at 1000 mg/L in methanol, and diluted with deionised water or acetonitrile as required for RP-LC and HILIC analyses, respectively. Calibration ranges spanned 1.25 – 40 mg/L for PCs and 2.5 – 80 mg/L for PDs and gallated PCs, with 6 concentration points each. For HILIC analyses, calibration samples were injected twice for MS calibration: 10 µL (full loop), followed by 1.5 µL (partial loop with needle overfill). For RP-LC, 3 µL was injected. All calibration samples were injected in duplicate.

FLD infusion experiments

To determine the optimum excitation and emission wavelengths for PAC standards, epicatechin (0.04 µM), epicatechin gallate (2.51 µM), epigallocatechin gallate (3.75 µM) and epigallocatechin (2.74 µM) solutions in methanol were infused into the flow cell of a Hewlett Packard 1046A programmable fluorescence detector. The scan functionality of Chemstation software (Agilent) was used to scan excitation and emission wavelengths to determine optimum values for each.

To investigate the influence of mobile phase composition on FLD response, each standard was diluted in mixtures of 0.1% formic acid and 0.1% formic acid (n = 10, in increments of 10% organic modifier). The response was measured using the optimised emission and excitation wavelengths for each compound.

Phloroglucinolysis

The phloroglucinol solution was prepared by adding 0.2 g ascorbic acid and 1.0 g phloroglucinol to a 10 mL volumetric flask and then filling to the mark with 0.2 N HCl in methanol. 100 µL wine or grape seed extract was allowed to react with 100 µL phloroglucinol solution at 50°C for 20 min, after which 1 mL aqueous sodium acetate solution (40 mM) was added to stop the reaction.

The procyanidin cleavage products were analysed by RP-LC on a 1260 Agilent system operated with Chemstation software. Two Chromolith Performance RP-18e columns (100 × 4.6 mm) columns were connected in series. The mobile phases comprised 1% (v/v) acetic acid (A) and 1% acetic acid (v/v) in acetonitrile (B). The following gradient was used: 3% B (6 min), 3 - 18% B (6 – 21 min), 80% B for 3 min before returning to initial conditions for a 3 min re-equilibration step. A flow rate of 2 mL/min was used at 30°C. 23.8 µL was injected and detection was performed at 280 nm. In order to determine the mean degree of polymerisation (mDP) of PACs, the mean ratio of the extension units to the terminal units was calculated. Calibration curves (ten points, 0.2 – 500 mg/L) were constructed for catechin and epicatechin to enable quantification of terminal and extension units according to [22].

3.3. RESULTS AND DISCUSSION

3.3.1 Selection of standard compounds and detection parameters

One of the major challenges in the accurate quantification of condensed tannins is the lack of commercially available standards. In the current work, a range of PACs belonging to different classes were either purchased commercially ((-)-epicatechin, (-)-epigallocatechin, (-)-epigallocatechin gallate and (-)-epicatechin gallate) or, preparatively isolated (PCs of DP 2-5). For the latter compounds, the predominant isomers from cocoa were isolated. In using these compounds as references for calibration and quantification, it is assumed that response factors do not vary significantly between PCs of the same DP. While little information is available in literature in support of this, the fact that catechin and epicatechin show identical response factors in UV and FLD points to the likely validity of this assumption for B-type PCs. UV detection and quantification of all PACs was performed at 280 nm, the wavelength of maximum absorption for all flavan-3-ol-derivatives [60, 86]. For MS detection, a Q-TOF instrument was used, which allowed attainment of accurate mass and tandem MS data for the identification of high MW PACs, as well as enhanced selectivity due to the relatively high resolving power of the TOF [74]. ESI was used in the negative ionisation mode, as this has been shown to be more sensitive than positive ionisation for PACs, and often results in less fragmentation which improves the detection sensitivity of molecular ions [75–77]. Regarding fluorescence detection, the selection of excitation (λ_{exc}) and emission (λ_{em}) wavelengths was less straightforward, in particular for the gallocatechins and the gallated PACs. In several previous studies which have used FLD for the detection of the latter compounds, the same excitation and emission wavelengths as used for PCs [30,47,78] were employed. However, to our knowledge their FLD properties of these classes of PACs have not yet been systematically investigated. For this reason, solutions of each of the standard compounds were individually infused into the FLD cell, and excitation and emission wavelengths were scanned to establish their fluorescent properties. These experiments aimed to ascertain sets of wavelengths where each of the target classes shows the greatest response in terms of sensitivity and selectivity.

The optimal wavelengths for PCs were in accordance with literature [47,78,79], at λ_{exc} 230 nm and λ_{em} 320 nm. Furthermore, the same wavelengths were optimal for the detection of gallocatechins, although these compounds were characterised by much lower response factors. Gallated PACs could be detected using the same wavelengths, while a second emission wavelengths (λ_{ex} 230 nm, λ_{em} 360 nm) showed better selectivity (but not sensitivity). Again, the response factors at both sets of wavelengths were much lower than for PCs (see further). Both sets of wavelengths were therefore used for calibration and sample

analysis, and the gallated PACs were then quantified using the determined set of wavelengths (λ_{ex} 230 nm, λ_{em} 360 nm).

Secondly, while the effect of mobile phase composition on UV and ESI-MS response factors are relatively well known, this is not the case for FLD detection of PACs. This aspect is of relevance in the current study due to the divergent mobile phases used in HILIC and RP-LC. Initial experiments were therefore performed to ascertain the effect of mobile phase composition on the FLD RFs of PCs. For this purpose, a range of concentrations for each standard in a range of mobile phase compositions (A/B, where A is 0.1% formic acid and B is 0.1% formic acid in acetonitrile) were infused into the detection cells and the response factors for each compound determined as a function of mobile phase composition; the results are summarised for selected PCs in **Figure 3.2**. From this figure it evident that within the range of mobile phase compositions used in HILIC separation of PCs (75-95%B), the response factors do not vary significantly. On the other hand, for typical RP-LC separations (2-30%B), RFs vary by up to 38% for the studied PCs. While this is an important observation, which points to the need for compound-specific calibration, it is likely less of a practical constraint than could be concluded from **Figure 3.2**. The reason for this is that most PCs of the same DP (or class) generally elute within a relatively small window of mobile phase composition (see further). This implies that RFs for wine tannins will typically vary by less than 10% in RP-LC. For these reasons, the effect of mobile phase composition (and by extension retention time) on FLD response was not taken into account in the current study. Another relevant observation is that lower response factors were measured for PCs in RP-LC compared to HILIC mobile phases, which has clear implications for the relative sensitivity of these separation modes in combination with FLD.

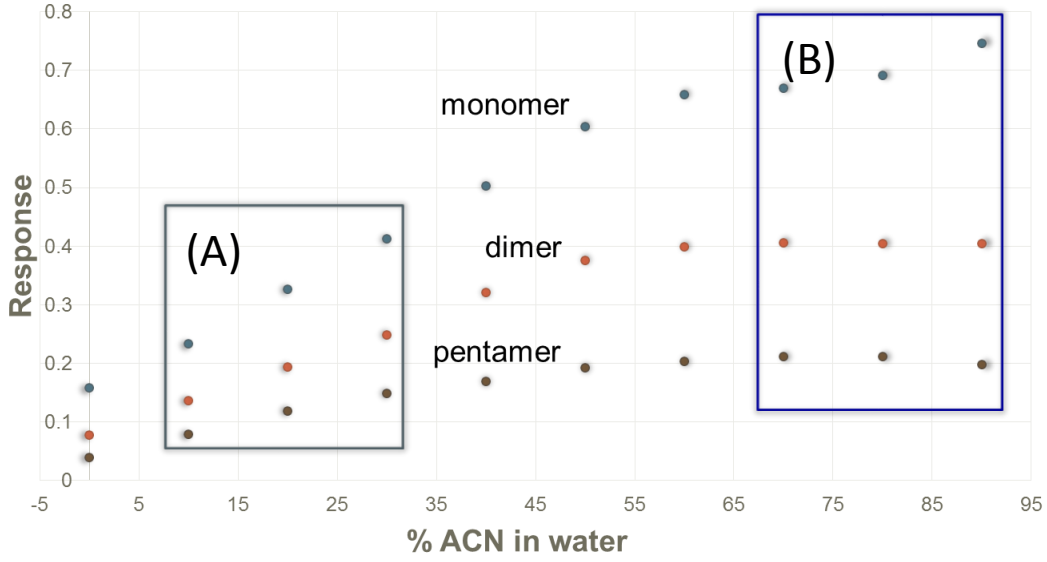


Figure 3.2: Variation in the fluorescence response factors of selected procyanidins (DP 1, 2 and 5) as a function of % organic modifier. (A) shows the window where compounds elute in RPLC and (B) the HILIC elution window. Experiments performed by infusion into the FLD cell. (λ_{exc} 230 nm, λ_{em} 320 nm).

Much more significant than the effect of mobile phase, is the decrease in RF as a function of DP. This observation is in line with previous reports regarding RRFs for FLD detection of PCs [5,61]. Furthermore, the same observation was made regarding RRFs for PCs with UV detection at 280 nm (**Figure S3, SI**, see further). These findings confirm the significant error associated with quantification of higher MW PCs as catechin/epicatechin equivalents (at least in m/v terms): this would result in severe underestimation of higher DP compounds [5,61,69].

3.3.2 Optimisation of HILIC and RP-LC separations and identification of compounds

HILIC has been shown to offer a useful method for the separation of PACs according to DP in a range of samples. Most of this work was performed on Diol phases [21,73,77,80]. In the current work, a BEH Amide phase was used, since this phase has previously been found to show good separation efficiency for anthocyanins and anthocyanin-tannin derivatives [81,82]. Indeed, using a generic HILIC grading comprising acidified acetonitrile/water mobile phases, good separation of PACs was obtained (**Figures 3.3** and 3.**4**). The characteristic HILIC retention order – i.e. increasing retention as a function of DP – was also observed on this phase [83,84]. However, in contrast to HILIC separation on Diol phases, some degree of isomeric separation was obtained on the Amide phase. Clear elution windows were observed for PACs of each DP (illustrated in **Figure 3.4A** for grape seed PACs). For PACs of the same DP, PCs eluted first, followed by PDs and then gallated PACs (**Table S1**)**.** These findings are in agreement with those of Kalili et al. [19], and can be ascribed to the increasing polarity of compounds as a function of hydroxylation and galloylation.

For the RP-LC separation, a superficially porous column was selected because of the excellent chromatographic performance of these phases at relatively modest pressures [85,86], which has also been confirmed for PACs [84]. Method optimisation was performed using generic RP-LC mobile phases, and provided good separation of PACs in grape seeds and wines (**Figures 3.3B).** Typical separation according to hydrophobicity provides isomeric separation for PACs **(Figure 3.4B)** [19,73,87]. In the reversed-phase separation, PDs were the first to elute, followed by PCs and then gallated PCs of the same DP (**Table S2**).

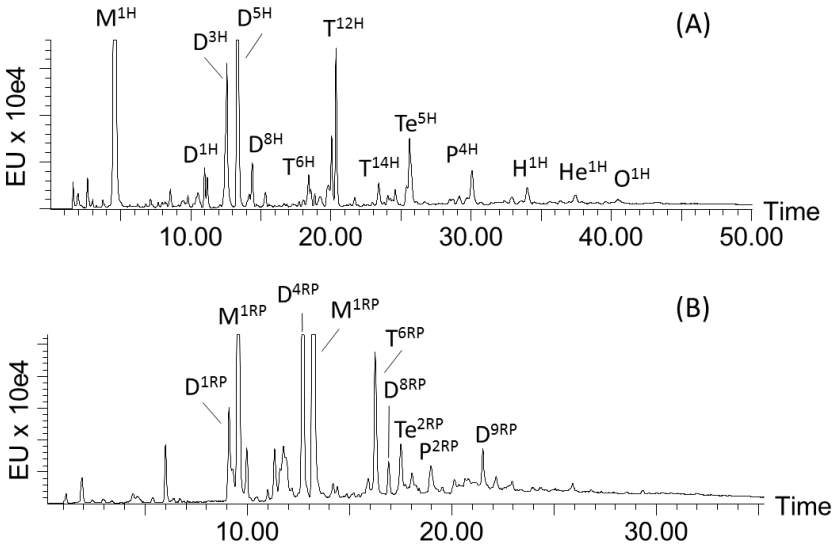


Figure 3.3: Representative FLD (λ_{exc} 230 nm, λ_{em} 320 nm) chromatograms obtained for the (A) HILIC-FLD and (B) RP-LC-FLD analyses of grape seed PACs. Labels indicate the compounds identified by MS and correspond with Tables S1 & 2: M = monomer, D = dimer, T = trimer, Te = tetramer, P = pentamer, H = hexamer, He = heptamer and O = octamer. Superscripts indicate the isomer number, with H referring to HILIC and RP to RP-LC separation (isomer numbers do not agree between these modes). Refer to Section 3.2.3 for experimental details.

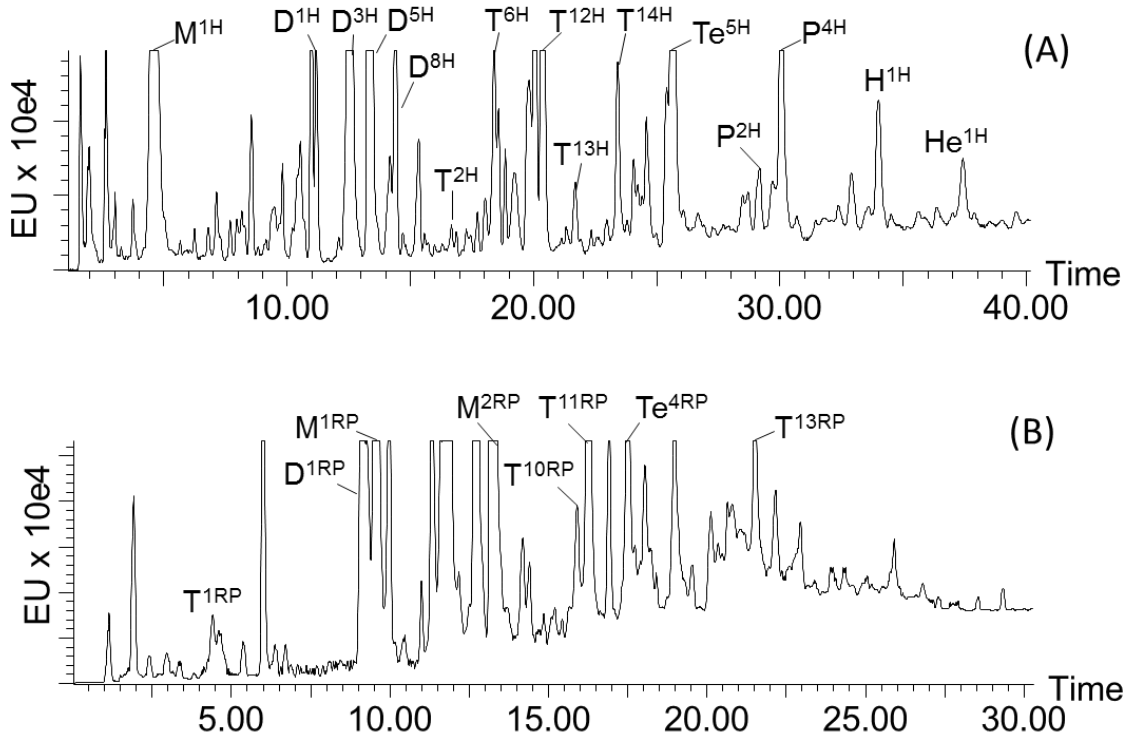


Figure 3.4: A detailed view of FLD chromatograms obtained for the (A) HILIC and (B) RP-LC separation of a grape seed extract. Peak labels correspond to Figure 3.3 and Tables S1 & S2.

For both HILIC and RP-LC separations, compounds were identified based on accurate mass as well as tandem mass spectral data obtained on Q-TOF instrument. For PACs of DP 4 and higher, multiply charged ions were detected as the base peak ions. From the high collision energy spectra obtained in MS^E mode, some information on the composition of each compound can be determined, based on the known fragmentation behaviour of PACs. Briefly, this entails retro-Diels-Alder fission of ring C, heterolytic ring fission (HRF) resulting in the loss of the A-ring, quinine methide (QM) cleavage of the interflavan-3-ol bond, and benzofuran-forming fission similar to HRF [20,88]. The fragmentation scheme and an example of the identification of a trimeric PAC are illustrated in **Figure S2**. Since this is not the main emphasis of the current contribution, further detailed discussion of the identification of compounds is omitted in the interest of brevity.

In total, 161 and 90 PACs were identified in this manner using the HILIC and RP-LC methods, respectively. The results are summarised in **Tables S1** (for HILIC) and **S2** (for RP-LC). For many compounds, large numbers of isomers were detected. For example, 9 PC dimers and 14 PC trimers were detected in HILIC (11 and 13 were detected in RP-LC). Often several of these could not be quantified due to their low levels. Note that due to the large numbers of isomers, assigning the corresponding isomers between HILIC and RP-LC separations was not possible; for this reason the compounds identified are differentiated by superscripts ^{H} and ^{RP} throughout this work. The majority of the compounds identified were detected in the grape seed samples, where PCs up to DP 10 and gallated PCs up to DP 7 were detected. In wine samples, gallotannins

up to DP 3, PCs up to DP 5 and gallated PCs up to DP 4 were detected. In general, the higher sensitivity of HILIC translated into detection of more high MW compounds in this mode compared to RP-LC.

3.3.3 Determination of relative response factors for proanthocyanins as a function of class and degree of polymerisation.

The optimised HILIC and RP-LC separations were hyphenated to diode array, FLD and Q-TOF-MS detectors in series and calibration was performed for each standard at six concentration levels (1.25 – 40 ppm for PCs and PDs, and 2.5 – 80 ppm for gallated PCs). Calibration in UV was performed at 280 nm, while both sets of wavelengths were used for FLD. For MS, calibration curves constructed using extracted ion chromatograms (EICs) for the base peak ion, multiply charged ion and/or dimeric ion, as relevant. Calibration curves for each of the standard compounds and detectors are presented in the **Supporting Information** (**Figures S3-S7**). The calibration data as well as preliminary data for limits of detection (LODs) and quantification (LOQs), determined using signal-to-noise ratios of 3:1 and 10:1, respectively, are summarised in **Tables 3.1** and **3.2**. From the calibration data, the RRFs for PCs of DP 1-5, as well as monomeric PDs and gallated PCs, could be obtained for UV, FLD and MS detection. This is pertinent, since RRFs, once reliably established, can be used to quantify PACs for which standards are not available based on calibration data for selected reference standards [17,55]. RRFs were determined in both molar and ppm (mg/L) units, denoted RRF_M and RRF_{ppm}, respectively, as reported in **Tables 3.1** and **3.2**.

For the PCs, the only compounds for which data for different DPs were available, the RRFs as a function of DP for the standard compounds (DP 1-5) were fit to exponential equations, for molar and ppm units. These were then used to extrapolate to higher DPs to obtain 'estimated' RFs for these compounds (mainly detected in grape seeds). The results are summarised for UV, FLD and MS data in **Figures 3.5, 3.6** and 3.**8**, respectively. Note that RRF values obtained in HILIC are presented in these figures, although similar values were determined for all three detectors in RP-LC (**Tables 3.1** and 3.**2**). This indicates that, although RF vary significantly with the mobile phase composition (*cf.* **Figure 3.2**), RRFs remain relatively unaffected.

The obtained RRFs were compared with those reported by Lin et al. [55] and Prior and Gu [5] for UV data (**Figure 3.5**), and with those of Hurst et al. [61] and Prior and Gu [5] for FLD data (**Figure 3.6**). Lin et al. [55] predicted that the UV molar RFs for PCs were additive, i.e. increased from 1 for monomers, 2 for dimers, etc. This was based on experimental data for PCs of DP 1-3 (orange data points in **Figure 3.5**). The measured RRF_M values for commercial standards did not increase linearly, although a vacuum dried trimer did fit this trend (black square in **Figure 3.5**) [55]. In contrast, our data indicate that the relative molar response deviates quite significantly from a linear trend (**Figure 3.5B**). One possible explanation of this

discrepancy is that the isolated PCs were not completely dry, despite being subjected to freeze-drying for at least 24 h. Another possible cause might lie in the three-dimensional structures that higher DP PCs assume in solution, which is related to their conformational and rotational movements. For PCs of DP 2 and 3, it has been shown that 3D structures can vary significantly of between different isomers [89,90], with trimers especially adopting compact conformations where stacking interactions between the phenolic rings are favoured [91]. Such phenomena almost certainly influence the molar extinction coefficients of the molecules, although to what extent is not certain in the absence of experimental data. These findings proved somewhat inconclusive in terms of the RRF_M of PCs with UV detection, which might require further investigation using pure and vacuum dried standards of DP > 3. Nevertheless, the trend in increasing RRF_M as a function of DP is certain, again highlighting the error associated with quantification of PCs using molar RFs for monomeric standards.

Regarding the FLD RRFs, our data are generally in accordance with those of Hurst et al. [61] who reported data for PCs of DP 2-10 isolated from cocoa. In both studies, RRF_{ppm} values were found to decrease exponentially with DP, whereas RRF_M values initially increased and then steadily declined (**Figure 3.6**). The generally good agreement between our data, obtained for individual isomers, and those reported in [61] for a mixture of isomers of the same DP support the relative accuracy of the FLD RRF values.

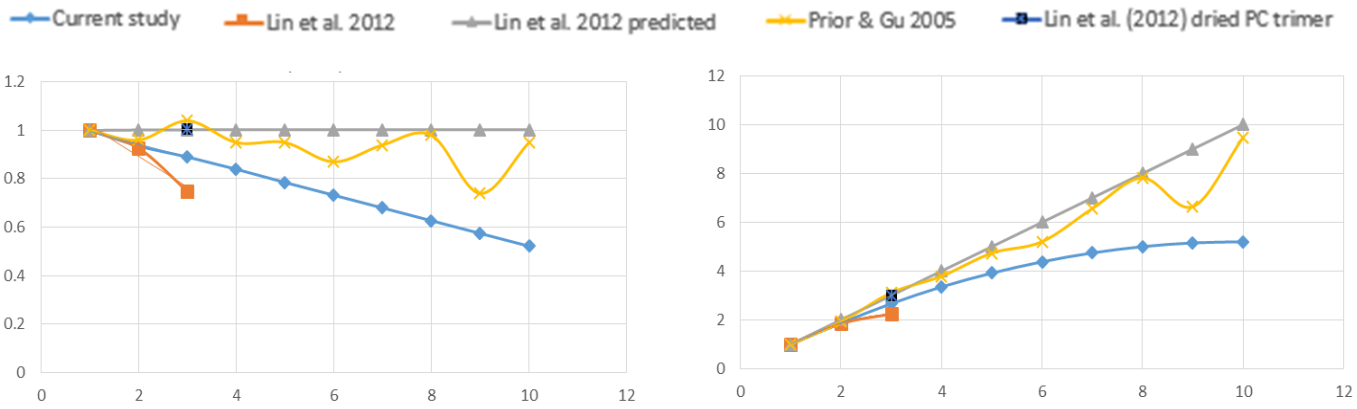


Figure 3.5: Relative UV response factors for PCs as a function of DP: (A) presents RRF_{ppm} values and (B) RRF_M data. Values presented are those from the current study, extrapolated from DP 5 to DP 10 (blue diamonds), predicted (grey triangles) and experimental (orange squares for standards) data taken from Lin et al. [55] and experimental data (yellow crosses) taken from Prior & Gu [5]. The black square indicates the values obtained by Lin et al. *[55]* for a vacuum-dried trimeric PC standard.

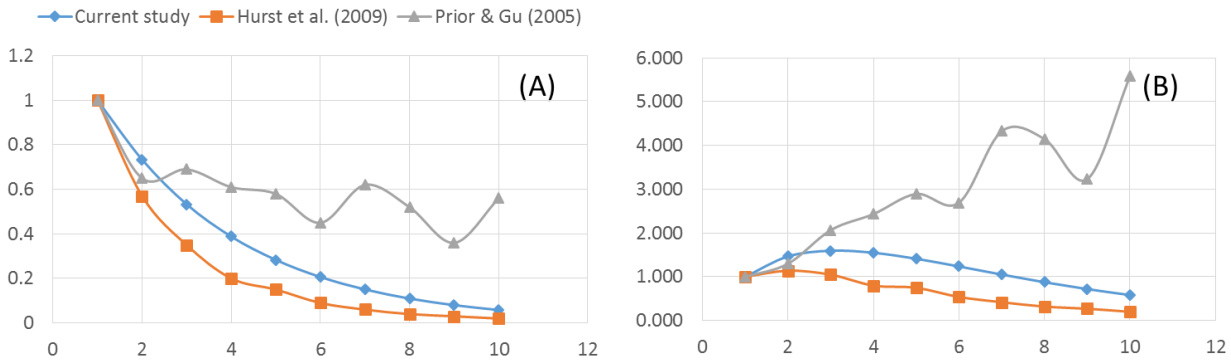


Figure 3.6: Relative FLD response factors for PCs as a function of DP: (A) presents RRF_{ppm} values and (B) RRF_M data. Values from the current study (obtained in HILIC), extrapolated from DP 5 to DP 10 (blue diamonds) are compared to those reported by Prior & Gu [5] (grey triangles) and Hurst et al. [61] (orange squares).

Calibration by MS detection is challenging due to the formation of several charged species for each compound in ESI. For the lower MW compounds (DP < 3), singly charged ions were observed in the mass spectra, while for higher MW multiply charged ions became predominant. For calibration purposes the base peak ions were used, i.e. the molecular ion for lower MW compounds and doubly charged species for DP 4-5. However, the linear dynamic range was limited to below 5 ppm for the HILIC separation of low DP (≤3) PCs (**Figure S6, SI**); saturation of the detector occurred beyond this level. Furthermore, the formation of dimeric ($[2M-H]^-$) ions for low MW compounds at high concentrations further limited the linear range for these compounds (**Figure 3.7A, B**). For this reason, two injection volumes (10 (full loop) and 1.5 µL (partial loop with needle overfill)) were used for HILIC calibration by MS. Examples of the calibration curves obtained for PCs in HILIC are presented in **Figure S6**, from which it is evident that full loop injections resulted in significant saturation (data were fit with a quadratic equation). For each compound, the linear range for full loop injections were also determined (typically up to a maximum concentration of 5 ppm), and this calibration curve was used to quantify compounds falling within this range by full loop injections. Partial loop injections (**Figure 3.6B**, secondary axis in **Figure S6C**) provided linear responses for all compounds up to 20 or 40 ppm, and these calibration curves were used to quantify compounds falling in the concentration range above the linear operating range for full loop injections. Of course, this approach implies also that a second injection of samples had to be performed in order to quantify all compounds. For RP-LC separations, the lower MS RFs resulted in linear calibration curves up to 20 or 40 ppm (**Figure S7**).

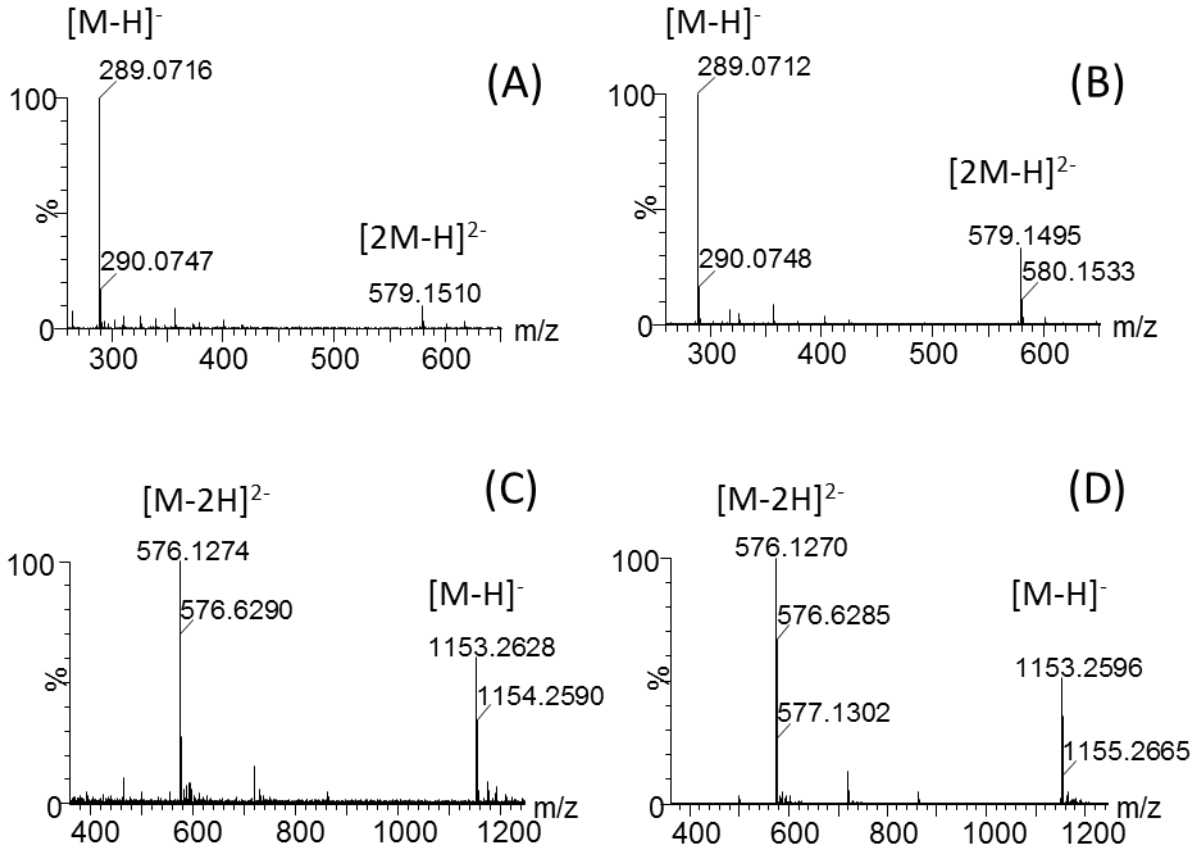


Figure 3.7: Examples of ESI mass spectra obtained for epicatechin (m/z 289) at (A) 2.5 ppm and (B) 40 ppm, demonstrating the differential formation of dimeric ions. (C) and (D) show the ESI mass spectra of a tetrameric PC (m/z 1153) at 1.25 ppm and 40 ppm, respectively. In this case the singly and doubly charged species vary in their relative ratios.

The experimental and extrapolated ESI-MS RRFs for PCs are presented in **Figure 3.8** for the relevant calibration curves. Similar to FLD, an exponential decrease in RRF_{ppm} is observed for the MS data, while RRF_M initially increases and then decreases with DP. In the case of ESI-MS, this observation is likely at least partially due to the formation of multiply charged species, the relative prevalence of which increases with DP.

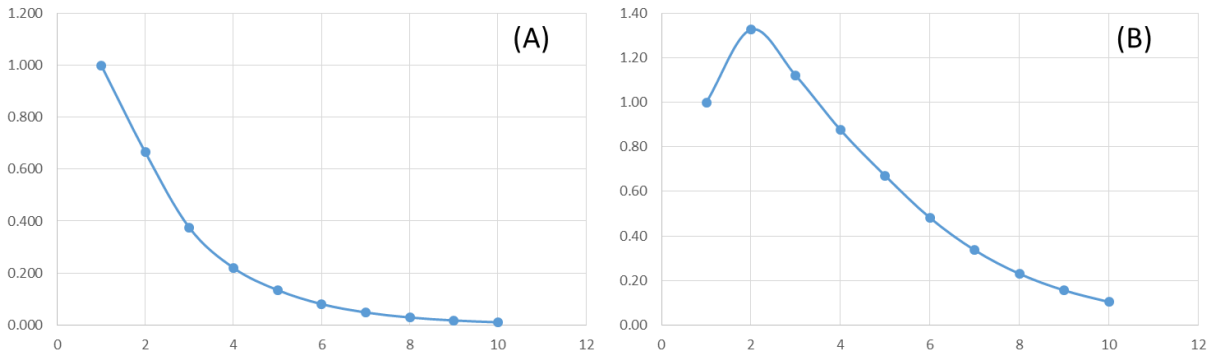


Figure 3.8: Relative (HILIC) MS response factors of the base peak ions of PCs as a function of DP, extrapolated from DP 5 to DP 10: (A) RRF_{ppm} and (B) RRF_M.

For the PDs and gallated PCs, for which higher DP standards were not available, the following approaches were used for the extrapolation of response factors to higher DP compounds. For gallated PCs, quantification was performed by UV and MS, using the approach for UV detection proposed by Lin et al. [55]. According to this, the UV RRF_M value for gallic acid was taken as 2.8 (compared to catechin) [17,55], and this value was added for the number of galloyl groups to that of the relevant PC, for which the RRF_M values reported in **Tables 3.1** and **3.2** and **Figures 3.5** and **3.7** were used. For RRF_M in MS, it was assumed that the same variation in response would occur, therefore the same approach was used, i.e. 2.8 RRF was added to that of the relevant PCs for quantification.

For gallotannins, the RRF_M values compared to catechin were both found to be 0.21 (**Tables 3.1** and **3.2**), roughly in accordance with the UV RRF_M value reported by Lin et al. [55]. For higher DP PACs containing (epi)gallocatechin units, the number of gallocatechin units were therefore represented by RRF_Ms of 0.3 and added to the RRF_M value for the corresponding PC unit (**Figure 3.8**). Clearly these assumptions are associated with potentially significant errors, but in the absence of more reliable data for higher DP compounds of these classes, this approach arguably provides the best approximation.

Table 3.1: A detailed summary of the HILIC calibration data.

HILIC	***UV***						***FLD***						***MS***						
Compound	**range (ppm)**	**r^2**	**LOQ**	**LOD**	**RRF$_{ppm}$**	**RRF_M**	**range (ppm)**	**r^2**	**LOQ**	**LOD**	**RRF$_{ppm}$**	**RRF_M**	**range[a]**	**range[b]**	**r^2**	**LOQ**	**LOD**	**RRF$_{ppm}$**	**RRF_M**
PC monomer	1.25 - 40.0	0.999	1.03	0.31	1	1.00	1.25 - 40.0	0.996	0.01	0.00	1.00	1.00	1.25 - 5.0	1.25 - 20.0	0.977	0.07	0.02	1.00	1.00
PC dimer	1.25 - 40.0	0.999	9.09	2.73	0.94	1.87	1.25 - 40.0	0.995	0.13	0.04	0.73	1.46	1.25 - 5.0	1.25 - 20.0	0.996	3.40	1.02	0.66	1.33
PC trimer	1.25 - 40.0	0.999	2.38	0.71	0.63	2.66	1.25 - 40.0	0.999	0.05	0.01	0.55	1.59	1.25 - 5.0	1.25 - 20.0	0.991	1.20	0.36	0.39	1.12
PC tetramer	1.25 - 40.0	0.999	2.14	0.64	0.84	3.35	1.25 - 40.0	0.999	0.05	0.01	0.39	1.55	1.25 - 5.0	1.25 - 20.0	0.990	1.03	0.31	0.22	0.88
PC pentamer	1.25 - 40.0	0.999	4.61	1.38	0.47	3.91	1.25 - 40.0	0.999	0.10	0.03	0.22	1.41	1.25 - 5.0	1.25 - 20.0	0.994	0.93	0.28	0.17	0.67
epigallocatechin	2.5 - 80.0	0.999	5.75	1.72	0.21	0.22	2.5 - 80.0	0.997	0.37	0.11	0.04	0.04	2.5 - 10.0	2.5 - 40.0	0.996	0.24	0.07	0.91	0.96
epicatechin gallate	2.5 - 80.0	0.999	0.30	0.09	3.70	5.65	2.5 - 80.0	0.996	4.44[a]	1.33[a]	0.00	0.01	2.5 - 10.0	2.5 - 40.0	0.972	-	-	0.61	0.93
epigallocatechin gallate	2.5 - 80.0	0.999	0.75	0.23	0.78	1.23	2.5 - 80.0	0.999	10.64[a]	3.19[a]	0.00	0.00	2.5 - 10.0	2.5 - 40.0	0.995	0.83	0.25	0.49	0.78

[a] Different set of wavelengths (λ_{exc} 230 nm, λ_{em} 360 nm) used for gallated PCs calibration

range[a]: linear range for full loop injection (10 µL) in MS.

range[b]: linear range for partial loop injection (3 µL) in MS.

Abbreviations: LOQ: limit of quantification in ppm, LOD: limit of detection in ppm, RRF_{ppm} : relative response factor in ppm, RRF_M: molar relative response factor.

Table 3.2: A detailed summary of the RP-LC calibration data.

RP-LC	*UV*						*FLD*						*MS*					
Compound	**range (ppm)**	**r^2**	**LOQ**	**LOD**	**RRF_{ppm}**	**RRF_M**	**range (ppm)**	**r^2**	**LOQ**	**LOD**	**RRF_{ppm}**	**RRF_M**	**range (ppm)**	**r^2**	**LOQ**	**LOD**	**RRF_{ppm}**	**RRF_M**
PC monomer	1.25 - 40.0	0.994	1.84	0.55	1	1.00	1.25 - 40.0	0.996	0.03	0.009	1.00	1.00	1.25 - 40.0	0.998	1.09	0.33	1.00	1.00
PC dimer	1.25 - 40.0	0.999	1.78	0.53	0.96	1.92	1.25 - 40.0	0.999	0.05	0.015	0.61	1.22	1.25 - 40.0	0.999	0.93	0.28	0.92	1.83
PC trimer	1.25 - 40.0	0.999	3.09	0.93	0.78	2.33	1.25 - 40.0	0.999	0.09	0.028	0.37	1.12	1.25 - 40.0	0.999	1.93	0.36	0.33	0.99
PC tetramer	1.25 - 40.0	0.997	3.85	1.15	0.8	3.19	1.25 - 40.0	0.999	0.12	0.037	0.38	1.50	1.25 - 40.0	0.998	3.72	1.12	0.32	1.27
PC pentamer	1.25 - 40.0	0.985	8.56	2.57	0.44	2.19	1.25 - 40.0	0.999	0.27	0.08	0.18	0.91	1.25 - 40.0	0.972	2.62	0.78	0.34	1.69
epigallocatechin	2.5 - 80.0	0.996	7.34	2.2	0.23	0.24	2.5 - 80.0	0.999	0.77	0.23	0.04	0.04	2.5 - 80.0	0.999	1.87	0.56	0.84	0.88
epicatechin gallate	2.5 - 80.0	0.961	1.91	0.57	1.42	2.17	2.5 - 80.0	0.939	16.61[a]	4.98[a]	0.00	0.00	2.5 - 80.0	0.999	3.19	0.96	0.66	1.01
epigallocatechin gallate	2.5 - 80.0	0.985	1.59	0.48	1.68	2.66	2.5 - 80.0	0.989	19.98[a]	5.99[a]	0.00	0.00	2.5 - 80.0	1.00	1.00	1.01	0.83	1.31

[a] Different set of wavelengths (λ_{exc} 230 nm, λ_{em} 360 nm) used for gallated PCs calibration
Abbreviations: LOQ: limit of quantification in ppm, LOD: limit of detection in ppm, RRF_{ppm} : relative response factor in ppm, RRF_M: molar relative response factor.

3.3.4 Comparison of UV, FLD and MS detectors for the quantification of condensed tannins

Comparing each of the three detectors from the perspective of quantification of condensed tannins, UV was the least sensitive and selective mode of detection (**Tables 3.1** and **3.2, Figures 3.9-3.11**). The lack of specificity of UV detection for PACs implies that chromatographic separation of the target compounds is critical. Coupled to the extreme complexity of wine and grape condensed tannins, which were not all separated in HILIC or RP-LC, the consequence is that quantification by UV should be treated with caution, as numerous instances of co-elution were observed, leading to overestimation of these compounds (refer also to **Section 3.5**). This is also the case for FLD, although to a lesser extent due to the higher selectivity of this mode of detection.

FLD was the most sensitive detector for PCs, with LOQs for monomers in HILIC and RPLC of 0.013 ppm and 0.031 ppm, respectively. Sensitivity was generally better in HILIC than RP-LC for all PCs owing to the effect of mobile phase composition on RF (*cf.* **Figure 3.2**). Note that this is despite the fact that HILIC separations were performed on a 4.6 mm i.d. column, whereas RP-LC separations performed at a lower flow rate on a 2.1 mm column. Further improvement in the UV and FLD sensitivity in HILIC may thus be achieved by reducing the column diameter.

For PDs and gallated PACs, however, the sensitivity of the FLD was lower than the UV at both sets of excitation/emission wavelengths. The LOQs for (epi)gallocatechin, (epi)catechin gallates and (epi)gallocatechin gallate were 5.75, 0.29 and 0.75 ppm, respectively, for UV detection, whereas the corresponding values for FLD were 0.37, 4.44 and 10.6 ppm, respectively (**Table 3.1**). **Figure 3.9** shows an example of the relative responses for each of the detectors for selected compounds, where epicatechin gallate was detected by both the Q-TOF-MS and PDA (280 nm) detectors but not by the FLD. For the quantification of gallated tannins, UV detection in combination with MS was therefore essential. On the other hand, the selectivity and sensitivity of both FLD and MS are much better than UV for the detection of PCs (**Figure 3.9C**).

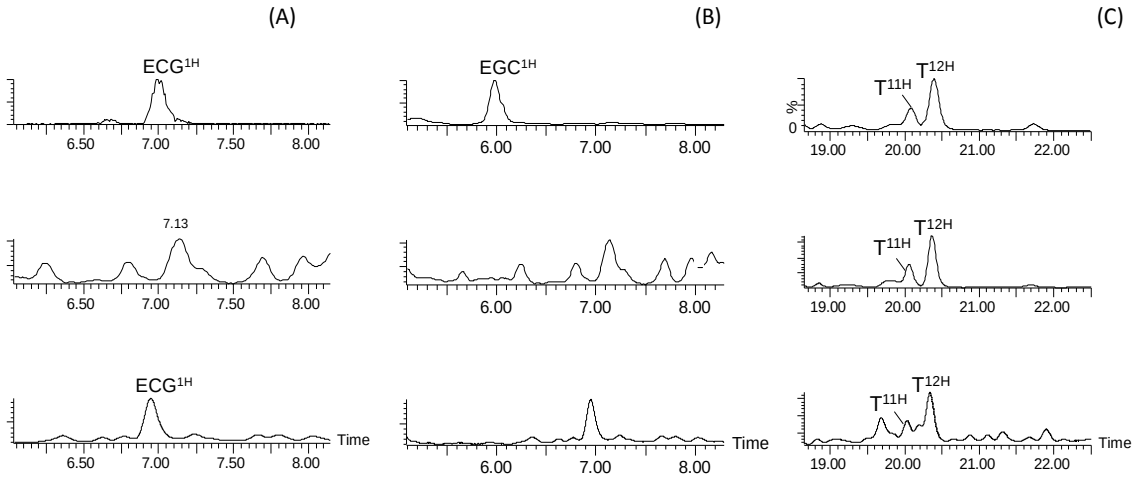


Figure 3.9: Chromatograms for (A) gallated PCs, (B) PDs and (C) PCs showing the sensitivity of each of the detectors. The top trace shows the EIC for the relevant compounds, the middle trace the FLD (λ_{exc} 230 nm, λ_{em} 320 nm) chromatogram and the bottom trace shows the UV (280 nm) chromatogram.

The MS was the most selective of the detectors, as the target analytes could be extracted from the total ion chromatogram, eliminating co-eluting compounds. Examples of the extracted ion chromatograms for trimeric PCs in HILIC and RP-LC are presented in **Figure S8**. In this regard the use of a high resolution mass spectrometer is essential, as this increases the specificity and allows distinguishing between isobaric compounds. However, for individual isomers chromatographic separation is of course essential to allow their detection and quantification. The most important benefit of MS is therefore for compound identification, which none of the spectroscopic detectors are capable of doing in the absence of standards (which, for the majority of identified PACs, are not commercially available). In this context it is also worth pointing out that confirmation of compound identity by MS is essential before quantification by UV or FLD can be performed. As a consequence, the LOQs for both these modes of detection are in essence tied to the LODs obtained by MS. Fortunately, the sensitivity of ESI-MS is relatively good, with LODs ranging from 0.02 - 1.02 ppm and 0.33 - 1.128 ppm in HILIC and RP-LC, respectively (**Tables 3.1** and **3.2**). Of course, once the retention times of the target PACs are established for a wide range of samples, it would be possible to quantify compounds based on retention time data alone, although this places and obvious premium on retention reproducibility. Aside from the MS being used to identify the compounds in the samples, quantification was also performed with Q-TOF.

3.3.5 Application to the quantitative analysis of grape seed and red wine tannins

Examples of the HILIC- and RP-LC-UV-FLD-MS chromatograms obtained for the analysis of grape seed and wine extracts are depicted in **Figures 3.10** and **3.11**, respectively. Differences in the relative selectivity of each of the detectors are clear from these chromatograms, as discussed above. Compounds were quantified using the

calibration data outlined in **Tables 3.1 and 3.2** and **Sections 3.3.3** and **3.3.4**, and quantitative data for the grape seed extract and each of the 9 wine samples analysed are presented in **Tables S3-S22** in the **Supporting Information**. In the interpretation of these results, it is important to consider the relative selectivities of each of the detectors. For example, PD derivatives and gallated PACs were generally only quantified by means of MS in most of the wine samples because of the relatively low levels of these compounds and the limited sensitivity of UV and FLD detectors for these compound classes. In grape seeds, where PDs were detected using all three detectors, there are some discrepancies between quantitative data because of differences in the number of compounds that could be quantified using each. The same is true to some extent for the PCs, although generally the isomers that could not be quantified using one of the detectors were present at low levels. The following discussion will focus on the PCs, as these represent the largest proportion of the quantified condensed tannins in grape seeds and wine and were quantified using all three detectors, which allows comparison of their performance. The total concentration of PCs in each of the analysed samples is presented in **Table 3.3**. Overall there was relatively good agreement between the quantitative data for FLD and MS in both HILIC and RP-LC methods, with UV often showing an overestimation of PC levels. This can be ascribed to the fact that co-elution was often observed, which affects quantitative data for UV detection due to the low selectivity of the technique. Furthermore the agreement between HILIC and RP-LC data for the same samples is also promising.

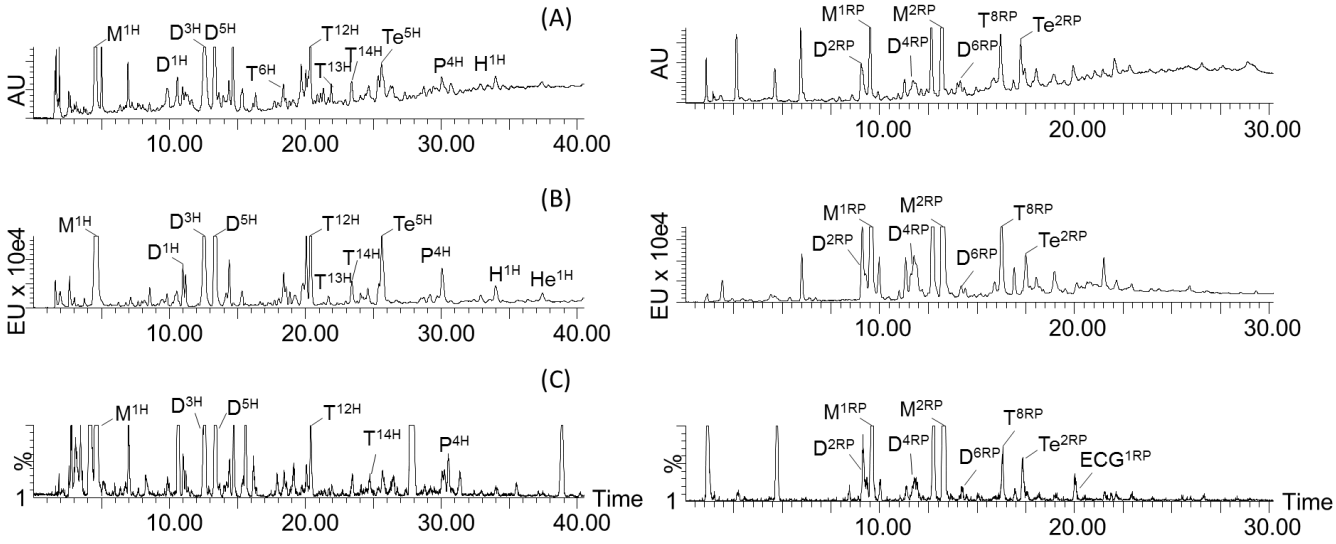


Figure 3.10: UV (280 nm, A), FLD (λ_{exc} 230 nm, λ_{em} 320 nm, B) and base peak ion (C) chromatograms for a grape seed extract (Tables S2and S3). The left-hand side shows the HILIC separation and the right-hand side the RP-LC separation. Peak labels correspond to Tables S1 and S2. Injection volume: 10 µL in HILIC and 3 µL in RP-LC.

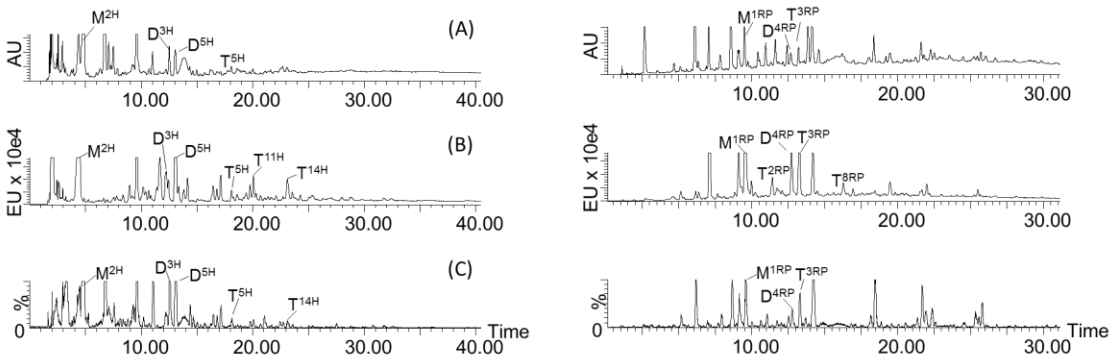


Figure 3.11: UV (280 nm, A), FLD (λ_{exc} 230 nm, λ_{em} 320 nm, B) and base peak ion (C) chromatograms for a red wine sample (sample Wine A, Tables S4 and S5). The left-hand side shows the HILIC separation and the right-hand side the RP-LC separation. Peak labels correspond to Tables S1 and S2. Injection volume: 10 µL in HILIC and 3 µL in RP-LC.

Table 3.3: Total concentrations (ppm) of PCs determined in wine and grape seed samples using the HILIC and RP-LC separations in combination with UV, FLD and MS detection. For detailed information on the individual components identified in each samples, refer to **Tables S3-S22**. mDP: mean degree of polymerisation as determined by phloroglucinolysis.

	HILIC			RPLC			
SAMPLE	UV	FLD	MS	UV	FLD	MS	mDP
WINE A	105	77.0	35.1	74.4	67.1	53.4	4.0
WINE B	88.1	57.0	56.6	69.3	52.5	43.9	2.3
WINE C	20.1	25.0	28.2	42.4	32.1	26.0	2.7
WINE D	84.2	112	127	156	137	110	3.1
WINE E	43.1	52.1	55.7	109	70.6	44.2	3.0
WINE F	20.8	19.1	12.0	24.1	18.4	15.8	2.4
WINE G	28.2	20.2	12.4	25.8	21.2	17.7	2.6
WINE H	24.5	23.2	14.5	31.3	26.7	20.2	2.5
WINE I	25.0	17.9	8.56	42.4	21.4	22.3	2.3
SEEDS	530	436	443	479	406	439	5.9

Figure 3.12 shows the correlations between quantitative data for the PCs (all samples) obtained by each separation method and each detector. As confirmed by **Figures 3.12A-C**, good agreement was observed for the data from each detector between HILIC and RP-LC separations, with the partial exception of UV detection, which showed significant differences for some samples. As alluded to above, this mostly due to overestimation of some compounds due to incomplete separation; the compounds not chromatographically resolved clearly differ between HILIC and RP-LC. From the data in **Table 3.3**, it is clear that this occurs for different samples in different modes, so that neither separation method is clearly superior. Both FLD and MS show good correlation between the data obtained by HILIC and RP-LC, which provides strong evidence for the validity of both chromatographic methods.

Comparing the different detectors in HILIC (**Figures 3.12D-F**) and RP-LC (**Figures 3.12G-I**), the overestimation of PC levels by UV is confirmed by the slopes which are significantly less than unity in the plots of UV vs. MS and FLD data. In contrast, the correlation between the FLD and MS data are good, with slopes close to 1 obtained for both separation modes. These correlations again support the applicability of these detectors used in series for the analysis of grape and wine condensed tannins.

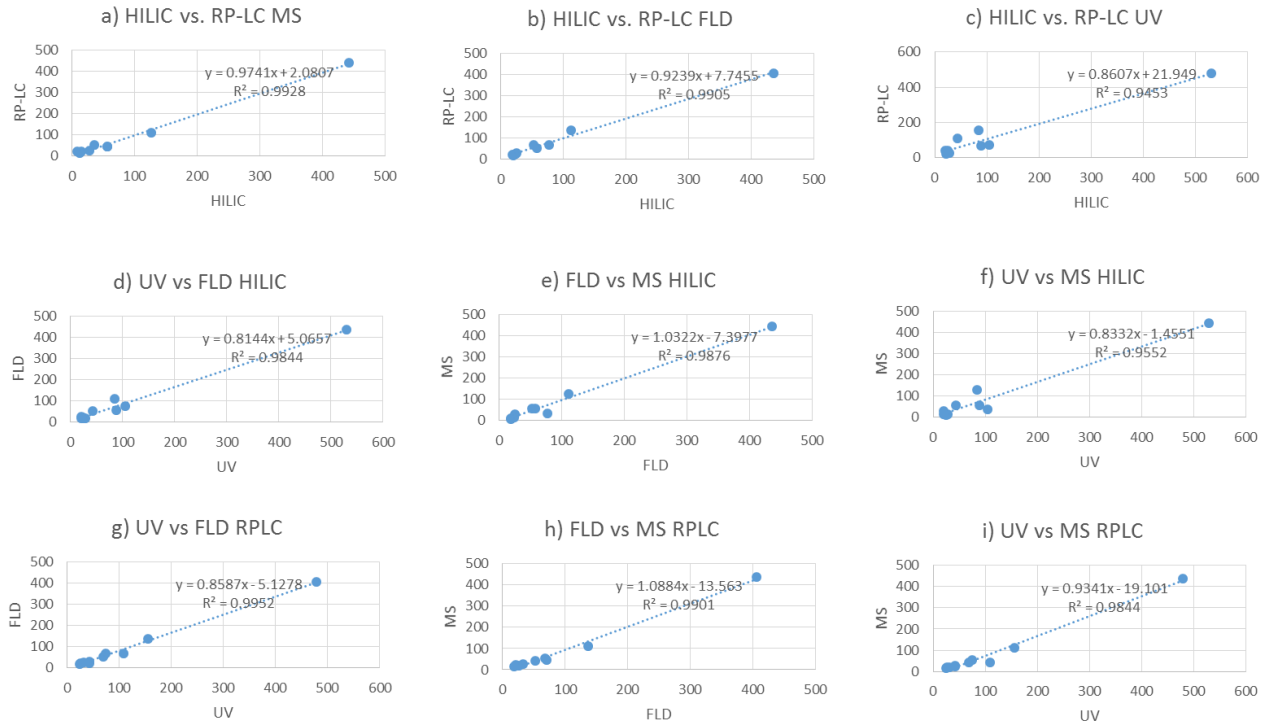


Figure 3.12: Line graphs showing the correlations, for PC quantification, between (A) HILIC and RPLC MS detection, (B) HILIC and RPLC FLD detection, (C) HILIC and RPLC UV detection. (D) – (F) show the correlations between the various detectors in HILIC, while (G) – (I) show the correlations between the various detectors in RPLC.

In the current study, a total of 161 PACs were detected in the wine and seed samples collectively using the HILIC method (most of these compounds were detected in the seed sample). Due to the slightly lower sensitivity of the RP-LC method, 90 compounds were identified using this method. These numbers compare favourably with the 115 compounds identified in grape seeds by HILIC×RP-LC separation by Kalili et al. [19], and 76 PACs detected in grape seed extracts by Lin et al. [17]. Of the identified compounds, 75 and 41 were quantified using the HILIC and RP-LC methods, respectively, in combination with all three detectors. Lin et al [17] reported quantitative data for 64 compounds in grape seeds using HR-MS data. PACs of DP up to 10 were identified in the current work, and quantitative data were obtained for compounds up to DP 7. In contrast, Lin et al. [17] only detected and quantified compounds up to a DP of 4 in grape seeds. Taken together, these results confirm the extreme complexity of grape and wine condensed tannins, and thereby the challenges associated with their accurate quantification.

The seed sample was found to have the highest concentration of tannins (~400 ppm, compared to ~20-150 for the wine samples), in agreement with previous studies [21,47,92–94]. This observation is in line with the fact that tannins are only partially extracted from the seeds present during the vinification process of red wines [95]. Rinaldi et al. [31] found that gallated PCs in particular were not easily extracted from seeds during winemaking. Only PCs and gallated PCs were detected in seed samples, whereas PDs were absent, in accordance with previous studies [23,47,64,92,96]. The mean degree of polymerisation (mDP) for the grape seed extract was determined as 5.9 using phloroglucinolysis (**Table 3.3**), which is in broad agreement with the compounds identified in this sample. While total tannins have been quantified in seeds in and wine using a variety of methods, the data are often not comparable (for example, precipitation and UV based methods generally indicate higher values than obtained by HPLC methods [43]). Nevertheless, comparing the values obtained in this work with literature data for individual components, generally good agreement is obtained for the levels of PCs and gallated PCs in grape seeds [16,17,94,97].

For the wine samples, the mDP values were generally much lower, ranging between 2.3 and 4. This is in agreement with the compounds identified and quantified in each sample, with the wine sample (A) containing the most high MW compounds (up to DP 5 PCs and mixed PC/PDs) also characterised by the highest mDP. Gallated PCs were only detected in one sample. The low prevalence of gallated PCs in wine have been reported before [47]. In contrast to the seed sample, however, PDs were detected in the wine samples (up to DP 5 in one sample), with monomers and dimers detected in most of the wines. The PDs present in wines originate from the skins of red grapes, where they have been previously detected [14,23,31,98]. Again, quantitative data for oligomeric PACs in wine are generally in agreement with values reported for a limited number of these compounds by HPLC in literature [30,99].

Taken together, our data for wines and grape seeds point to the extreme complexity of the oligomeric PACs present in these samples. This complexity increases with increasing MW of the compounds, although quantitative data for such compounds reflect the fact that the levels of individual compounds are relatively low compared to the monomeric and dimeric species. However, due to the sheer number of these compounds, their contribution to the physiological and sensory properties may be significant. A further informative application of the methods reported here would be to investigate the evolution of oligomeric tannin levels as a function of wine age.

3.4. CONCLUSIONS

Despite the important contribution of condensed tannins to wine properties, comprehensive, accurate quantitative data for these compounds is still lacking due to a lack of suitable analytical methods and commercial standards. In this contribution, HILIC as well as RP-LC methods were developed and evaluated for the accurate quantification of condensed tannins in grape seeds and wine samples. Procyanidins of DP up to pentamers were isolated from cocoa and used as standards, together with selected commercial (epi)gallocatechins and gallated proanthocyanins. Relative response factors for each class of proanthocyanins were determined using each of the detectors, and these were extrapolated to higher MW compounds. High resolution mass spectral data were used to identify a total of 160 proanthocyanins in grape and wine samples, and compounds were quantified using UV, FLD and EIC data.

UV, as expected, was the least sensitive of the detectors, although this form of detection proved essential for the quantification of gallated proanthocyanins. FLD was the most sensitive of the detectors for procyanidins, and provided good quantitative data for this class. High resolution mass spectrometry plays a critical part in this multi-detector strategy by allowing identification of non-standard compounds, and also for the quantification of especially prodelphinidin derivatives and gallated proanthocyanins, despite the limited linear range of the detector which necessitated the use of two injections. The HILIC method was found to provide better sensitivity for all detectors compared to RP-LC, with similar chromatographic performance, and is thus the preferred mode of quantification of condensed tannins. Comparable quantitative data were obtained from the HILIC and RP-LC methods with FLD and MS detection for different wine samples and a grape seed extract. The combination of optimised chromatographic methods with UV, FLD and HR-MS detection in series therefore provides a promising approach for the accurate quantification of condensed tannins in complex matrices such as grape seeds and wine.

The current work shed new light on the complexity of wine tannins, and the challenges associated with their quantification. Further work should be done to confirm the accuracy of the relative response factors of each class of proanthocyanin and each detector, and to extend the range of compounds for which relative response factors are available. This would ideally allow to obtain accurate quantitative data using calibration data for a selected few standard compounds. Clearly, such a method(s) should then also be validated to demonstrate applicability to real-life samples. Once achieved, the availability of an accurate analytical method for the determination of condensed tannins will prove invaluable in exploring the chemistry of these important wine constituents.

3.5. References

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Supporting Information: Chapter 3

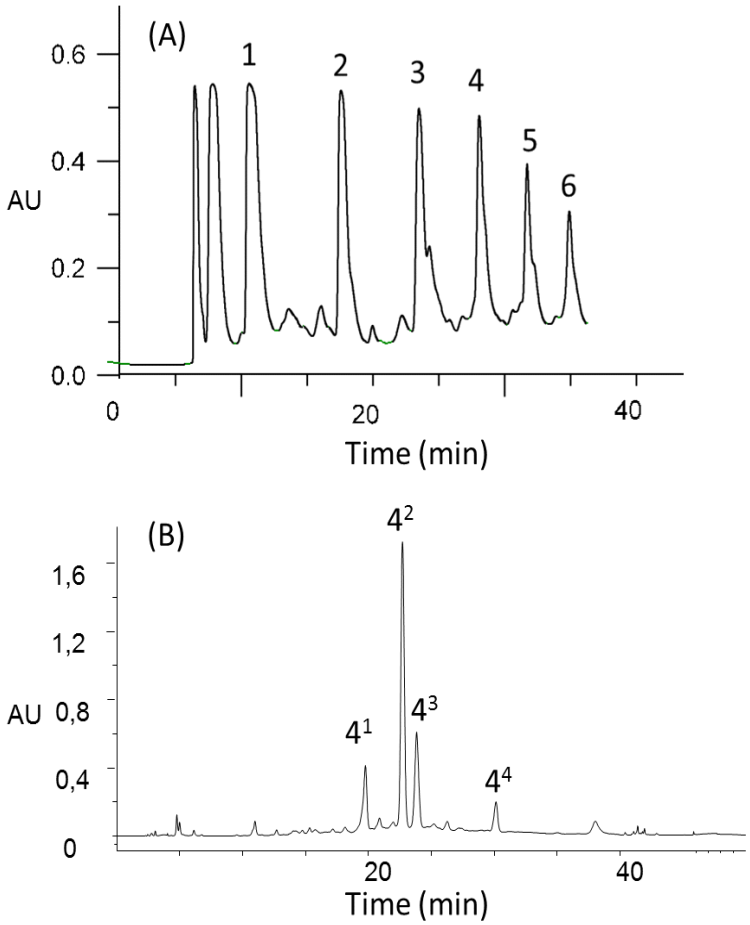

Figure S1: Examples of chromatograms obtained for the semi-preparative (A) HILIC and (B) RP-LC separation of cocoa PCs to obtain reference compounds. (B) shows the RP-LC separation of tetrameric PCs following isolation by HILIC. Labels 1-6 indicate PCs of the same DP, while superscripts in (B) distinguish between isomers of the same DP. For the tetrameric PCs, 4^2 was collected for use as a DP4 PC standard. For experimental conditions, refer to Section 3.2.2.

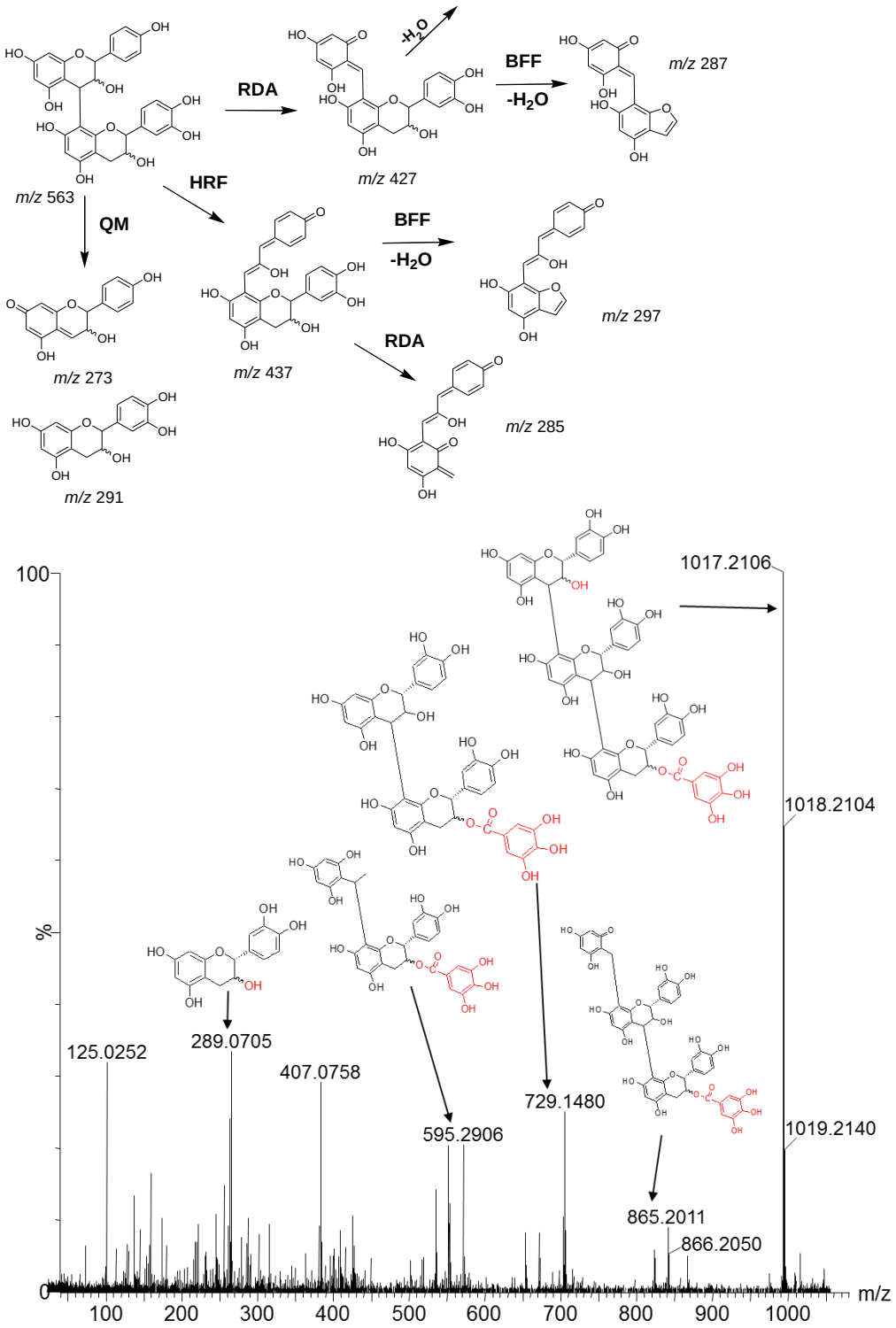

Figure S2: (Top) General fragmentation patterns of a dimeric proanthocyanidin in positive ionisation ESI-MS. Retro-Diels Alder (RDA), heterocyclic ring fission (HRF), quinone methide (QM) and benzofuran-forming fission (BFF) fragmentation patterns are demonstrated. Source: Reproduced from De Villiers et al. [1]. (Bottom) shows the high collision energy (MS^E) spectrum of a (epi)catechin-(epi)catechin)-(epi)catechin gallate trimeric proanthocyanin illustrating the corresponding fragmentation pathways.

Table S3: Summary of the ions detected by HILIC-ESI-MS analysis of condensed tannins in wine and seed extracts. Base peak ions are indicated with an asterisk.

Compound[a]	t_R (min)	samples	molecular formula	$[M - H]^-$ exp	$[M - H]^-$ theor	$[M - 2H]^{2-}$	$[M - 3H]^{3-}$	Δppm	MS^E fragments
monomer^{1H} (epicatechin)	4.56	seeds, wine	$C_{15}H_{14}O_6$	289.0715	289.0712	-		1.0	289.0713, 245.0812
monomer^{2H} (catechin)	4.65	seeds, wine	$C_{15}H_{14}O_6$	289.0711	289.0712			-0.3	289.0715, 245.0819
(epi)cat/(epi)cat dimer^{1H}	11.0	seeds, wine	$C_{30}H_{26}O_{12}$	577.1346	577.1346	-		0.0	577.1352*, 425.0872, 407.0771, 289.0718, 165.0337, 125.0249
(epi)cat/(epi)cat dimer^{2H}	11.2	seeds, wine	$C_{30}H_{26}O_{12}$	577.1351	577.1346			0.9	577.1342*, 425.0854, 407.0765, 289.0709, 165.0348, 125.0260
(epi)cat/(epi)cat dimer^{3H}	12.5	seeds, wine	$C_{30}H_{26}O_{12}$	577.1343	577.1346			-0.5	577.1342*, 425.0878, 407.0768, 289.0713, 125.0239
(epi)cat/(epi)cat dimer^{4H}	12.6	seeds	$C_{30}H_{26}O_{12}$	577.1345	577.1346			-0.2	577.1350*, 425.0866, 407.0760, 289.0709, 125.0240
(epi)cat/(epi)cat dimer^{5H}	12.9	seeds, wine	$C_{30}H_{26}O_{12}$	577.135	577.1346			0.7	577.136
(epi)cat/(epi)cat dimer^{6H}	13.3	seeds, wine	$C_{30}H_{26}O_{12}$	577.1355	577.1346			1.6	577.1356*, 425.0880, 407.0770, 289.0717, 125.0243
(epi)cat/(epi)cat dimer^{7H}	13.4	seeds, wine	$C_{30}H_{26}O_{12}$	577.1347	577.1346			0.2	577.1346*, 425.0875, 407.0767, 289.0713, 125.0247
(epi)cat/(epi)cat dimer^{8H}	13.7	seeds	$C_{30}H_{26}O_{12}$	577.1349	577.1346			0.5	577.1343, 407.0781*, 289.0705, 165.0343, 125.0258
(epi)cat/(epi)cat dimer^{9H}	14.0	seeds	$C_{30}H_{26}O_{12}$	577.1346	577.1346			0.5	577.1377*, 289.0726, 165.0269, 125.0262
(epi)cat/(epi)cat/(epi)cat trimer^{1H}	16.5	seeds	$C_{45}H_{38}O_{18}$	865.2005	865.198	-		2.9	-
(epi)cat/(epi)cat/(epi)cat trimer^{2H}	16.7	seeds	$C_{45}H_{38}O_{18}$	865.1959	865.198			-2.1	865.1968, 577.1351, 497.0787, 289.0710*, 125.0249
(epi)cat/(epi)cat/(epi)cat trimer^{3H}	16.9	seeds	$C_{45}H_{38}O_{18}$	865.1963	865.198			-2.0	865.1958, 577.1445, 497.0782, 289.0730*
(epi)cat/(epi)cat/(epi)cat trimer^{4H}	17.7	seeds	$C_{45}H_{38}O_{18}$	865.198	865.198			0.0	865.2023*, 715.1256, 577.1354, 407.0767, 289.0729, 125.0249
(epi)cat/(epi)cat/(epi)cat trimer^{5H}	18.3	seeds, wine	$C_{45}H_{38}O_{18}$	865.1965	865.198			-1.7	865.1992*, 577.1349, 407.0763, 289.0714, 125.0232
(epi)cat/(epi)cat/(epi)cat trimer^{6H}	18.4	seeds, wine	$C_{45}H_{38}O_{18}$	865.1966	865.198			-1.6	865.1960*, 713.1464, 577.1340, 407.0772, 289.0714, 125.0242
(epi)cat/(epi)cat/(epi)cat trimer^{7H}	18.6	seeds	$C_{45}H_{38}O_{18}$	865.1983	865.198			0.3	865.1969*, 713.1528, 577.1368, 407.0782, 289.0718, 125.0244
(epi)cat/(epi)cat/(epi)cat trimer^{8H}	18.9	seeds, wine	$C_{45}H_{38}O_{18}$	865.1987	865.198			0.8	865.1992*, 713.1452, 577.1303, 407.0765, 289.0711, 125.0251

(epi)cat/(epi)cat/(epi)cat trimer[9H]	19.3	seeds, wine	$C_{45}H_{38}O_{18}$	865.1969	865.198			-1.3	865.1983*, 713.1455, 577.1306, 407.0760, 289.0711, 125.0248
(epi)cat/(epi)cat/(epi)cat trimer[10H]	19.9	seeds, wine	$C_{45}H_{38}O_{18}$	865.1978	865.198			0.2	865.1960*, 713.1520, 577.1288, 407.0774, 289.0709, 125.0243
(epi)cat/(epi)cat/(epi)cat trimer[11H]	20.1	seeds, wine	$C_{45}H_{38}O_{18}$	865.1974	865.198			-0.7	865.1973*, 713.1525, 577.1343, 407.0763, 289.0710, 125.0242
(epi)cat/(epi)cat/(epi)cat trimer[12H]	20.4	seeds	$C_{45}H_{38}O_{18}$	865.1978	865.198			0.2	865.1972*, 713.1477, 577.1342, 407.0763, 289.0710, 125.0242
(epi)cat/(epi)cat/(epi)cat trimer[13H]	21.7	seeds, wine	$C_{45}H_{38}O_{18}$	865.199	865.198			1.2	865.1974*, 713.1503, 577.1326, 407.0769, 289.0719, 125.0252
(epi)cat/(epi)cat/(epi)cat trimer[14H]	23.3	seeds, wine	$C_{45}H_{38}O_{18}$	865.1972	865.198			-0.9	865.1974*, 713.1475, 577.1322, 407.0760, 289.0714, 125.0244
(epi)cat/(epi)cat/(epi)cat/(epi)cat tetramer[1H]	23.8	seeds, wine	$C_{60}H_{50}O_{24}$	1153.2614	1153.2614	576.1271*		0.0	1153.2453, 865.1919, 575.1198, 407.0774, 289.0722, 125.0259*
(epi)cat/(epi)cat/(epi)cat/(epi)cat tetramer[2H]	24.1	seeds, wine	$C_{60}H_{50}O_{24}$	1153.2596	1153.2614	576.1269*		-1.6	1153.2577, 865.1874, 577.1357, 575.1170, 407.0780, 289.0717, 125.0256*
(epi)cat/(epi)cat/(epi)cat/(epi)cat tetramer[3H]	24.6	seeds, wine	$C_{60}H_{50}O_{24}$	1153.2626	1153.2614	576.1270*		1.0	1153.2588, 865.1988, 577.1344, 575.1180, 407.0762, 289.0707, 125.0244*
(epi)cat/(epi)cat/(epi)cat/(epi)cat tetramer[5H]	24.9	seeds, wine	$C_{60}H_{50}O_{24}$	1153.2567	1153.2614	576.1257*		-4.1	1153.2710, 863.1674, 577.1315, 575.1180, 407.0782, 289.0720, 125.0239*
(epi)cat/(epi)cat/(epi)cat/(epi)cat tetramer[4H]	25.4	seeds, wine	$C_{60}H_{50}O_{24}$	1153.2612	1153.2614	576.1268*		-0.2	1153.2583, 863.1730, 577.1236, 575.1174, 407.0749, 289.0714, 125.0240*
(epi)cat/(epi)cat/(epi)cat/(epi)cat tetramer[6H]	25.7	seeds, wine	$C_{60}H_{50}O_{24}$	1153.2587	1153.2614	576.1269*		-2.7	1153.2594*, 865.1929, 863.1826, 577.1313, 575.1182, 407.0767, 289.0709, 125.0239
(epi)cat/(epi)cat/(epi)cat/(epi)cat tetramer[7H]	26.7	seeds, wine	$C_{60}H_{50}O_{24}$	1153.259	1153.2614	576.1221*		-2.1	1154.2723, 865.1749, 575.1107, 289.0734, 125.0255*
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat pentamer[1H]	29.1	seeds, wine	$C_{75}H_{62}O_{30}$	1441.3265	1441.3248	720.1590		1.2	-
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat pentamer[2H]	29.2	seeds, wine	$C_{75}H_{62}O_{30}$	1441.319	1441.3248	720.1596		-4.0	1441.3137, 863.1838, 577.1320, 575.1180, 407.0767, 289.0713*, 125.0241
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat pentamer[3H]	29.7	seeds, wine	$C_{75}H_{62}O_{30}$	1441.3131	1441.3248	720.1597		-8.1	1441.3157, 863.1776, 577.1331, 575.1208, 407.0774, 289.0713*, 125.0245
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat pentamer[4H]	30.1	seeds	$C_{75}H_{62}O_{30}$	1441.3198	1441.3248	720.1598		-3.5	1441.3242, 863.1868, 575.1194, 407.0761, 289.0699*, 125.0250
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat hexamer[1H]	32.4	seeds	$C_{90}H_{74}O_{36}$	1730.529	-	864.1886	-	-	864.1893, 575.1201, 407.0785, 289.0708*, 125.0249

(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat hexamer^{2H}	33.0	seeds	$C_{90}H_{74}O_{37}$	1730.529		864.1888		-	864.1893, 575.1201, 407.0785, 289.0708*, 125.0249
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat hexamer^{3H}	34.0	seeds, wine	$C_{90}H_{74}O_{38}$	1730.529		864.1913		-	864.1893, 575.1201, 407.0785, 289.0708*, 125.0249
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat/ (epi)cat heptamer^{1H}	36.4	seeds	$C_{105}H_{86}O_{42}$	2018.4594	-	1008.2212	-	-	863.1926, 577.1345, 575.1165, 407.0770, 289.0717, 287.0555*, 125.0240
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat/ (epi)cat heptamer^{2H}	37.5	seeds	$C_{105}H_{86}O_{43}$	2018.4594		1008.2236		-	865.1971, 863.1915, 577.1347, 575.1198, 407.0785, 289.0718, 287.0560*, 125.0243
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat octamer^{1H}	39.6	seeds	$C_{120}H_{98}O_{48}$	2306.5228	-	1152.2517	-	-	863.1749, 577.1338, 407.0774, 289.0714, 287.0563*, 125.0238
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat octamer^{2H}	40.5	seeds	$C_{120}H_{98}O_{49}$	2306.5228		1152.2368		-	863.1885, 575.1197, 407.0794, 287.0565*, 125.0243
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat nonamer	43.3	seeds	$C_{135}H_{110}O_{54}$	2594.5861	-	1296.781	-	-	863.1870, 575.1202, 407.0782, 287.0551*, 125.0244
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat/ (epi)cat decamer	45.8	seeds	$C_{150}H_{122}O_{60}$	2882.6495	-	1440.8065	960.2039	-	575.1182, 407.0770, 287.0566, 125.0243
(epi)gallocat^{1H}	5.98	seeds, wine	$C_{15}H_{14}O_7$	305.0662	305.0661	-		0.3	305.0665*, 255.2303, 183.0182, 149.0420, 125.0262
(epi)gallocat^{2H}	6.62	wine	$C_{15}H_{14}O_7$	305.0667	305.0661			2.0	305.0661*, 191.203, 179.0352, 149.0097, 125.0242
(epi)cat/(epi)gallocat dimer^{1H}	15.2	wine	$C_{30}H_{26}O_{13}$	593.1296	593.1295	-		0.2	593.1324*, 407.0787, 289.0699, 125.0247
(epi)cat/(epi)gallocat dimer^{2H}	15.9	wine	$C_{30}H_{26}O_{13}$	593.1291	593.1295			-0.7	593.1344, 407.0766, 289.0721, 125.0245*
(epi)cat/(epi)gallocat dimer^{3H}	16.4	wine	$C_{30}H_{26}O_{13}$	593.1292	593.1295			-0.5	593.1280*, 407.0759, 305.0659, 289.0706, 125.0252
(epi)cat/(epi)gallocat dimer^{4H}	16.5	wine	$C_{30}H_{26}O_{13}$	593.1294	593.1295			-0.2	593.1299*, 305.0670, 289.0724, 287.0557, 245.0461, 125.0255
(epi)cat/(epi)gallocat dimer^{5H}	16.8	wine	$C_{30}H_{26}O_{13}$	593.1299	593.1295			0.7	593.1295*, 425.0877, 407.0776, 289.0715, 125.0252
(epi)cat/(epi)gallocat dimer^{6H}	17.1	wine	$C_{30}H_{26}O_{13}$	593.1292	593.1295			-0.5	593.1288*, 425.0869, 407.0769, 289.0716, 177.0197, 125.0250

(epi)gallocat dimer[1H]	17.6	wine	$C_{30}H_{26}O_{14}$	609.1229	609.1244		-2.5	609.1327, 577.1297, 305.0666, 289.0715, 287.0588, 177.0203, 125.0254
(epi)gallocat dimer[2H]	20.6	wine	$C_{30}H_{26}O_{14}$	609.1246	609.1244		0.3	609.1270*, 577.1356, 575.1315, 441.0801, 423.0722, 305.0667, 289.0735, 287.0563, 177.0202, 125.0245
(epi)gallocat dimer[3H]	21.0	wine	$C_{30}H_{26}O_{15}$	609.1255	609.1244		1.8	609.1245*, 528.1720, 441.0832, 423.0710, 305.0683, 177.0200, 125.0252
(epi)cat/(epi)cat/(epi)gallocat trimer[1H]	20.8	wine	$C_{45}H_{38}O_{19}$	881.1902	881.1929	440.098	-3.2	881.1916, 423.0725, 289.0729, 125.0255*
(epi)cat/(epi)cat/(epi)gallocat trimer[2H]	21.4	wine	$C_{45}H_{38}O_{19}$	881.1908	881.1929	440.098	-2.4	881.1943, 865.1956*, 577.1331, 575.1183, 407.0762, 289.0722, 287.0581, 177.0194, 125.0247*
(epi)cat/(epi)cat/(epi)gallocat trimer[3H]	21.6	wine	$C_{45}H_{38}O_{19}$	881.1949	881.1929	440.098	2.3	881.1948, 729.1483, 577.1298, 575.1182, 407.0785, 289.0724, 287.0565, 177.0197, 125.0246*
(epi)cat/(epi)cat/(epi)gallocat trimer[4H]	21.9	wine	$C_{45}H_{38}O_{19}$	881.1909	881.1929	440.098	-2.3	881.1856, 407.0762, 289.0709, 177.0198, 125.0253*
(epi)cat/(epi)cat/(epi)gallocat trimer[5H]	22.7	wine	$C_{45}H_{38}O_{19}$	881.1914	881.1929	440.098	-1.7	881.1990, 865.1829, 577.1364, 575.1164, 407.0766, 289.0736, 287.0579, 125.0254*
(epi)cat/(epi)cat/(epi)gallocat trimer[6H]	23.2	wine	$C_{45}H_{38}O_{19}$	881.1929	881.1929	440.098	0.0	881.1910, 865.1978*, 577.1332, 407.0767, 289.0715, 177.0204, 125.0252
(epi)cat/(epi)cat/(epi)gallocat trimer[7H]	23.6	wine	$C_{45}H_{38}O_{19}$	881.1909	881.1929	440.098	-2.3	881.1907, 577.1351, 407.0758, 289.0713, 177.0194, 125.0251*
(epi)cat/(epi)cat/(epi)gallocat trimer[8H]	25.2	wine	$C_{45}H_{38}O_{19}$	881.1924	881.1929	440.098	-0.6	881.1944, 577.1275, 407.0777, 289.0736*, 177.0202, 125.0259
(epi)cat/(epi)cat/(epi)gallocat trimer[9H]	26.8	wine	$C_{45}H_{38}O_{19}$	881.1894	881.1929	440.098	-4.0	881.1906, 577.1346, 575.1165, 407.0775, 289.0724, 177.0201, 125.0250*
(epi)cat/(epi)gallocat/ (epi)gallocat trimer[1H]	24.3	wine	$C_{45}H_{38}O_{20}$	897.1857	897.1878		-2.3	897.1981, 881.1888, 577.1346, 575.1142, 407.0748, 289.0721*, 287.0561, 177.0193, 125.0249
(epi)cat/(epi)gallocat/ (epi)gallocat trimer[2H]	25.1	wine	$C_{45}H_{38}O_{20}$	897.1899	897.1878		2.3	897.1980, 577.1334, 407.0807, 289.0719, 177.0206, 125.0251*
(epi)cat/(epi)gallocat/ (epi)gallocat trimer[3H]	25.6	wine	$C_{45}H_{38}O_{20}$	897.1865	897.1878		-1.3	897.1845, 577.1337, 289.0712, 177.0209, 125.0249
(epi)cat/(epi)gallocat/ (epi)gallocat trimer[4H]	26.4	wine	$C_{45}H_{38}O_{20}$	897.1851	897.1878		-3.0	897.1823, 577.1372, 407.0785, 289.0726, 177.0196, 125.0254*
(epi)cat/(epi)gallocat/ (epi)gallocat trimer[5H]	27.0	wine	$C_{45}H_{38}O_{20}$	897.1851	897.1878	228.0905	-3.0	897.1836, 407.0760, 289.0729, 177.0197, 125.0247*

epigallocat trimer[1H]	30.5	wine	$C_{45}H_{38}O_{21}$	913.1752	913.1827		8.2	913.1813, 577.1274, 575.1186, 407.0751, 289.0701, 177.0189, 125.0250*
(epi)cat/(epi)cat/(epi)gallocat/ (epi)gallocat tetramer[1H]	31.8	wine	$C_{60}H_{50}O_{26}$	1185.2498	1185.2512	592.5	-1.9	1185.2478, 577.1340, 407.0758, 289.0724, 177.0198, 125.0249*
(epi)cat/(epi)cat/(epi)cat/ (epi)gallocat tetramer[1H]	28.0	wine	$C_{60}H_{50}O_{25}$	1169.2532	1169.2563	584.5	-2.7	1169.2603, 865.1943, 863.1895, 713.1497, 577.1339, 575.1176, 407.0749, 289.0712, 177.0199, 125.0242*
(epi)cat/(epi)cat/(epi)cat/ (epi)gallocat tetramer[2H]	28.8	wine	$C_{60}H_{50}O_{25}$	1169.2563	1169.2563	584.5	0.0	1169.2550, 865.1915, 577.1301, 407.0772, 289.0723, 287.0577, 177.0201, 125.0251, 96.9603*
(epi)gallocat pentamer[1H]	32.9	wine	$C_{75}H_{62}O_{35}$	-	1521.2993	760.5	-	577.1337, 575.1158, 407.0746, 289.0725, 287.0565, 177.0195, 125.0252*, 96.9606
(epi)cat/(epi)cat/(epi)gallocat/ (epi)gallocat/(epi)gallocat pentamer[1H]	38.4	wine	$C_{75}H_{62}O_{33}$	-	1489.3095		-	577.1290, 575.1188, 407.0785, 289.0717, 287.0565, 177.0195, 125.0249*, 96.9613
(epi)cat/(epi)cat/(epi)gallocat/ (epi)gallocat/(epi)gallocat pentamer[2H]	39.0	wine	$C_{75}H_{62}O_{33}$	-	1489.3095		-	865.1654, 863.1772, 577.1390, 575.1240, 407.0746, 303.0512, 289.0726, 287.0578, 177.0197, 125.0247*, 96.9606
(epi)cat/(epi)cat/(epi)gallocat/ (epi)gallocat/(epi)gallocat pentamer[3H]	39.3	wine	$C_{75}H_{62}O_{33}$	1489.3180	1489.3095	744.1507*	5.7	863.1745, 577.1322, 423.0706, 407.0762, 289.0704, 287.0557, 177.0193, 125.0253*, 96.9608
(epi)cat/(epi)cat/(epi)gallocat/ (epi)gallocat/(epi)gallocat pentamer[4H]	39.9	wine	$C_{75}H_{62}O_{33}$	-	1489.3095		-	881.1817, 577.1364, 575.1169, 407.0775, 289.0275, 287.0555, 177.0198, 125.0250*, 96.9604
(epi)cat/(epi)cat/(epi)cat/ (epi)gallocat/(epi)gallocat pentamer[1H]	35.3	wine	$C_{75}H_{62}O_{32}$	1473.3035	1473.3146	736.1544*	-7.5	577.1351, 575.1202, 407.0749, 289.0731, 287.0571, 177.0194, 125.0248*, 96.9610
(epi)cat/(epi)cat/(epi)cat/ (epi)gallocat/(epi)gallocat pentamer[2H]	36.1	wine	$C_{75}H_{62}O_{32}$	-	1473.3146	736.1517*	-	577.1343, 575.1204, 407.0788, 289.0713, 287.0561, 177.0209, 125.0250*, 96.9612
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)gallocat pentamer[1H]	32.5	wine	$C_{75}H_{62}O_{31}$	1457.3024	1457.3197	728.1539*	-12	577.1327, 575.1173, 407.0769, 289.0728, 287.0566, 177.0204, 125.0240*, 96.9608
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)gallocat pentamer[2H]	33.3	wine	$C_{75}H_{62}O_{31}$	-	1457.3197	728.1538*	-	577.1373, 575.1226, 407.0752, 289.0705, 287.0575, 177.0200, 125.0253*, 96.9607

(epi)cat/(epi)cat/(epi)gallocat/ (epi)gallocat/(epi)gallocat/ (epi)gallocat hexamer[1H]	46.4	wine	$C_{90}H_{74}O_{40}$	-	1794.3756	896.1808*	-	577.1308, 575.1208, 423.0717, 407.0753, 287.0562, 177.0193, 125.0249*, 96.9600
(epi)cat/(epi)cat/(epi)gallocat/ (epi)gallocat/(epi)gallocat/ (epi)gallocat hexamer[2H]	46.9	wine	$C_{90}H_{74}O_{40}$	-	1794.3756	896.6750*	-	577.1408, 575.1187, 289.0728, 287.0548, 177.0188, 125.0252*, 96.9607
(epi)cat/(epi)cat/(epi)cat/ (epi)gallocat/(epi)gallocat/ (epi)gallocat hexamer[1H]	43.0	wine	$C_{90}H_{74}O_{39}$	-	1778.3807	896.1807*	-	577.1308, 575.1188, 423.0759, 405.0556, 287.0568, 177.0209, 125.0247*, 96.9618
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)gallocat/ (epi)gallocat hexamer[1H]	39.2	wine	$C_{90}H_{74}O_{38}$	-	1762.3858	880.1875*	-	577.1368, 575.1199, 407.0757, 289.0741, 287.0573, 177.0206, 125.0248*, 96.9608
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)gallocat hexamer[2H]	36.4	wine	$C_{90}H_{74}O_{37}$	-	1746.3909	872.6833*	-	577.1293, 575.1135, 407.0782, 289.0720, 287.0566, 177.0207, 125.0245*, 96.9614
(epi)catgallate[1H]	6.67	seeds	$C_{22}H_{18}O_{10}$	441.0817	441.0822	-	-1.1	441.0826, 289.0738, 169.0146, 125.0242*
(epi)catgallate[2H]	7.00	seeds	$C_{22}H_{18}O_{10}$	441.0811	441.0822		-2.5	441.0814*, 289.0713, 169.0139, 125.0239
(epi)cat/(epi)catgallate dimer[1H]	13.4	seeds	$C_{37}H_{30}O_{16}$	729.1459	729.1456		0.4	729.1455, 577.1349*, 407.0766, 289.0716, 125.0245
(epi)cat/(epi)catgallate dimer[2H]	13.7	seeds	$C_{37}H_{30}O_{16}$	729.1475	729.1456		2.6	729.1448, 577.1339, 407.0776*, 289.0710, 125.0250
(epi)cat/(epi)catgallate dimer[3H]	14.7	seeds, wine	$C_{37}H_{30}O_{16}$	729.1453	729.1456		-0.4	729.1454*, 577.1292, 559.0916, 441.0815, 407.0773, 289.0714, 169.0140, 125.0242
(epi)cat/(epi)catgallate dimer[4H]	15.3	seeds	$C_{37}H_{30}O_{17}$	729.1469	729.1456		1.8	729.1467*, 577.1358, 407.0787, 289.0719, 125.0255
(epi)cat/(epi)catgallate dimer[5H]	15.4	seeds, wine	$C_{37}H_{30}O_{17}$	729.1461	729.1456		0.7	729.1462*, 577.1340, 407.0774, 289.0723, 125.0247
(epi)catgallate/(epi)catgallate dimer[1H]	14.4	seeds	$C_{44}H_{34}O_{20}$	881.1585	881.1565		2.3	881.1586, 729.1466, 577.1288, 407.0782, 289.0722*
(epi)catgallate/(epi)catgallate dimer[2H]	16.4	seeds	$C_{44}H_{34}O_{20}$	881.1563	881.1565		-0.2	881.1602*, 729.1439, 577.1309, 559.1204, 541.1162, 407.0722, 289.0719, 169.0139, 125.0230
(epi)catgallate/(epi)catgallate/ (epi)catgallate trimer[1H]	20.6	seeds	$C_{66}H_{50}O_{30}$	1321.225	1321.2309	660.1099*	-4.5	577.1348, 575.1197, 407.0770*, 289.0708, 287.0551, 169.0136, 125.0246, 96.9677
(epi)catgallate/(epi)catgallate/ (epi)catgallate trimer[2H]	20.9	seeds	$C_{66}H_{50}O_{30}$	1321.224	1321.2309	660.1123*	-5.2	577.1316, 575.1219, 407.0757, 289.0693, 169.0139, 125.0237
(epi)catgallate/(epi)catgallate/ (epi)catgallate trimer[3H]	22.2	seeds	$C_{66}H_{50}O_{30}$	1321.2448	1321.2309	660.1158*	10.5	577.1351, 575.1205, 407.0791, 289.0717, 287.0559, 169.0317, 125.0224*, 96.9659

(epi)cat/(epi)catgallate/ (epi)catgallate trimer^{1H}	18.4	seeds	$C_{59}H_{46}O_{26}$	1169.2089	1169.2199	584.1055*	-9.4	1169.2211, 1017.2015, 865.1979*, 729.1369, 713.1490, 577.1327, 407.0768, 289.0705, 125.0235
(epi)cat/(epi)catgallate/ (epi)catgallate trimer^{2H}	19.6	seeds	$C_{59}H_{46}O_{26}$	1169.2239	1169.2199	584.1063*	3.4	1169.2161, 1017.2043, 865.1998, 713.1401, 577.1276, 575.1171, 407.0795, 289.0708*, 169.0108, 125.0246
(epi)cat/(epi)catgallate/ (epi)catgallate trimer^{3H}	19.7	seeds	$C_{59}H_{46}O_{26}$	-	1169.2199	584.1055*	-	1169.2124, 1017.2084*, 865.1984, 729.1341, 577.1315, 575.1176, 407.0775, 289.0706, 287.0557, 169.0137, 125.0241, 96.9691
(epi)cat/(epi)catgallate/ (epi)catgallate trimer^{4H}	20.2	seeds	$C_{59}H_{46}O_{26}$	-	1169.2199	584.1075*	-	1169.2073, 1017.2068*, 865.1951, 729.1426, 577.1335, 575.1199, 407.0764, 289.0717, 125.0236, 96.9684
(epi)cat/(epi)catgallate/ (epi)catgallate trimer^{5H}	20.7	seeds	$C_{59}H_{46}O_{26}$	1169.2133	1169.2199	584.1075*	-5.6	1169.2091, 1017.1941, 865.1964*, 729.1509, 713.1693, 577.1277, 575.1221, 407.0748, 289.0726, 169.0174, 125.0250, 96.9672
(epi)cat/(epi)catgallate/ (epi)catgallate trimer^{6H}	20.9	seeds	$C_{59}H_{46}O_{26}$	1169.218	1169.2199	584.1055*	-1.6	1169.2167, 1017.2000*, 865.1909, 713.1342, 577.1333, 575.1212, 407.0775, 289.0717, 169.0134, 125.0248, 96.9672
(epi)cat/(epi)catgallate/ (epi)catgallate trimer^{7H}	21.4	seeds	$C_{59}H_{46}O_{26}$	1169.2136	1169.2199	584.1053*	-5.4	1169.2267, 1017.2067, 865.1895, 727.1254, 575.1199, 407.0733, 289.0704, 169.0137, 125.0239*
(epi)cat/(epi)catgallate/ (epi)catgallate trimer^{8H}	22.2	seeds	$C_{59}H_{46}O_{26}$	-	1169.2199	584.1041*	-	1169.1967, 1153.2552, 1017.1960, 865.1927, 729.1459, 577.1339, 575.1204, 407.0767, 305.0545, 289.0720, 169.0137, 125.0242*, 96.9681
(epi)cat/(epi)catgallate/ (epi)catgallate trimer^{9H}	23.0	seeds	$C_{59}H_{46}O_{26}$	1169.2141	1169.2199	584.1055*	-5	1169.2109, 1153.2267, 1017.1994, 865.1945, 729.1281, 713.1418, 577.1339, 575.1181, 407.0763, 289.0688, 287.0540, 169.0142, 125.0232*, 96.9635
(epi)cat/(epi)cat/(epi)catgallate trimer^{1H}	17.8	seeds	$C_{52}H_{42}O_{22}$	1017.2065	1017.2089	508.1022	-2.4	1017.1883, 865.1945*, 729.1434, 713.1432, 577.1332, 289.0705, 287.0553, 125.0248, 96.9617
(epi)cat/(epi)cat/(epi)catgallate trimer^{2H}	19.7	seeds,wine	$C_{52}H_{42}O_{22}$	1017.2068	1017.2089	508.0991	-2.1	1017.2084*, 865.1984, 729.1427, 577.1315, 575.1174, 407.0781, 289.0707, 287.0555, 169.0136, 125.0239, 96.9688

(epi)cat/(epi)cat/(epi)catgallate trimer[3H]	19.9	seeds	$C_{52}H_{42}O_{22}$	1017.2082	1017.2089	508.1003	-0.7	1017.2085, 865.1971*, 577.1250, 575.1186, 407.0767, 289.0706, 287.0548, 169.0133, 125.0250, 96.9688
(epi)cat/(epi)cat/(epi)catgallate trimer[4H]	20.2	seeds	$C_{52}H_{42}O_{22}$	1017.2053	1017.2089	508.1001	-3.5	1017.2063, 865.1971*, 729.1427, 713.1488, 577.1340, 575.1202, 407.0758, 289.0713, 287.0566, 169.0136, 125.0252, 96.9691
(epi)cat/(epi)cat/(epi)catgallate trimer[5H]	20.9	seeds	$C_{52}H_{42}O_{22}$	1017.2075	1017.2089	508.0978	-1.4	1017.2037*, 865.1901, 729.1488, 577.1331, 575.1218, 407.0769, 289.0711, 169.0135, 125.0243
(epi)cat/(epi)cat/(epi)catgallate trimer[6H]	21.2	seeds, wine	$C_{52}H_{42}O_{22}$	1017.2096	1017.2089	508.1035	0.7	1017.2101*, 865.1816, 729.1401, 577.1298, 575.1213, 407.0753, 289.0720, 169.0115, 125.0239
(epi)cat/(epi)cat/(epi)catgallate trimer[7H]	21.4	seeds, wine	$C_{52}H_{42}O_{22}$	1017.2028	1017.2089	508.0989	-6	1017.2072*, 865.1868, 729.1396, 577.1337, 575.1172, 407.0758, 289.0707, 169.0136, 125.0235, 96.9672
(epi)cat/(epi)cat/(epi)catgallate trimer[8H]	22.0	seeds, wine	$C_{52}H_{42}O_{22}$	1017.2086	1017.2089	508.0997	-0.3	1017.2103*, 865.2018, 729.1467, 577.1352, 575.1197, 407.0762, 289.0706, 169.0134, 125.0253, 96.9648
(epi)cat/(epi)cat/(epi)catgallate trimer[9H]	22.5	seeds	$C_{52}H_{42}O_{22}$	1017.2031	1017.2089	508.0995	-5.7	1017.2097, 865.2108, 729.1426, 577.1346, 575.1220, 407.0764, 289.0718, 125.0242*, 96.9640
(epi)cat/(epi)catgallate/ (epi)catgallate/(epi)catgallate tetramer[1H]	25.3	seeds	$C_{81}H_{62}O_{36}$	-	1526.3173	804.1445*	-	1305.2792, 1153.2557, 1017.2073, 865.1776, 729.1422, 577.1337, 575.1184, 289.0719, 287.0559, 169.0135, 125.0241
(epi)cat/(epi)catgallate/ (epi)catgallate/(epi)catgallate tetramer[2H]	25.8	seeds	$C_{81}H_{62}O_{36}$	-	1526.3173	804.6492*	-	1305.2723, 1153.2589, 865.1906, 577.1329, 575.1192, 407.0768, 289.0708, 287.0551, 169.0155, 125.0238, 96.9624
(epi)cat/(epi)catgallate/ (epi)catgallate/(epi)catgallate tetramer[3H]	26.1	seeds	$C_{81}H_{62}O_{36}$	-	1526.3173	804.1419*	-	1442.3030, 1305.2692. 1153.2537, 863.1761, 729.1405, 575.1190, 407.0751, 289.0712*, 169.0142, 125.0239
(epi)cat/(epi)cat/(epi)catgallate /(epi)catgallate tetramer[1H]	23.4	seeds	$C_{74}H_{58}O_{32}$	-	1457.2833	728.1397*	-	1305.2706, 1153.2581, 865.1982*, 729.1451, 713.1470, 577.1304, 575.1176, 407.0757, 289.0721, 169.0140, 125.0247
(epi)cat/(epi)cat/(epi)catgallate /(epi)catgallate tetramer[2H]	23.9	seeds	$C_{74}H_{58}O_{32}$	-	1457.2833	728.1418*	-	1458.3008, 1305.2483, 865.1996, 729.1400, 577.1362, 575.1211, 407.0769, 289.0725, 125.0246*

(epi)cat/(epi)cat/(epi)catgallate /(epi)catgallate tetramer^{3H}	24.9	seeds	$C_{74}H_{58}O_{32}$	-	1457.2833	728.1371*	-	1458.2477, 1305.2601, 1153.2721, 1017.1294, 865.1698, 863.1909, 729.1478, 577.1355, 575.1181, 407.0757, 289.0710, 125.0243*, 96.9269
(epi)cat/(epi)cat/(epi)catgallate /(epi)catgallate tetramer^{4H}	25.4	seeds	$C_{74}H_{58}O_{32}$	-	1457.2833	728.1370*	-	1458.2748, 1305.2764, 1153.2589, 1017.2020, 865.1766, 729.1410, 575.1173, 407.0746, 289.0761, 169.0131, 125.0237*
(epi)cat/(epi)cat/(epi)catgallate /(epi)catgallate tetramer^{5H}	25.8	seeds	$C_{74}H_{58}O_{32}$	-	1457.2833	728.1358*	-	1458.2855, 1441.3196, 1305.2727, 1153.2628, 865.1912, 863.1900, 577.1323, 575.1185, 407.0766, 289.0716, 169.0145, 125.0239*, 96.9630
(epi)cat/(epi)cat/(epi)catgallate /(epi)catgallate tetramer^{6H}	26.4	seeds	$C_{74}H_{58}O_{32}$	-	1457.2833	728.1386*	-	1458.2863, 1305.2683, 1154.2714, 1017.2108, 865.1816, 729.1396, 577.1339, 575.1196, 407.0749, 289.0709*, 169.0151, 125.0241, 96.9602
(epi)cat/(epi)cat/(epi)catgallate /(epi)catgallate tetramer^{7H}	26.9	seeds	$C_{74}H_{58}O_{32}$	1457.2811	1457.2833	728.1360*	-1.1	1457.2701, 1305.2595, 1153.2494, 863.1901, 729.1472, 577.1296, 575.1172, 407.0719, 289.0711, 169.0138*, 125.0239, 96.9575
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer^{1H}	21.9	seeds, wine	$C_{67}H_{54}O_{28}$	-	1305.2723	652.1306*	-	1305.2736, 1153.2701, 1017.2091*, 865.2006, 729.1449, 577.1362, 575.1182, 407.0766, 289.0705, 287.0574, 169.0128, 125.0251
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer^{2H}	22.7	seeds, wine	$C_{67}H_{54}O_{28}$	1305.2703	1305.2723	652.1345*	-1.5	1305.2719, 1153.2650, 1017.2037, 865.1942, 729.1403, 577.1322, 407.0784, 289.0724, 169.0124, 125.0247*, 96.9664
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer^{3H}	23.5	seeds, wine	$C_{67}H_{54}O_{28}$	1305.2772	1305.2723	652.1331*	3.8	1305.2720, 1153.2677, 865.1978*, 577.1313, 407.0763, 289.0714, 125.0247
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer^{4H}	24.4	seeds, wine	$C_{67}H_{54}O_{28}$	1305.2803	1305.2723	652.1331*	6.1	1305.2728, 1153.2593, 1017.1876, 865.1961, 729.1393, 577.1355, 407.0780, 289.0715, 125.0250*, 96.9637
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer^{5H}	24.7	seeds, wine	$C_{67}H_{54}O_{28}$	1305.2657	1305.2723	652.1324*	-5.1	1305.2633, 1153.2516, 1017.1812, 863.1831, 729.1448, 575.1194, 407.0758, 289.0704, 169.0148, 125.0245*, 96.9669
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer^{6H}	25.4	seeds, wine	$C_{67}H_{54}O_{28}$	-	1305.2723	652.1318*	-	1305.2797, 1153.2573*, 1017.2020, 865.1776, 729.1408, 575.1184,

								407.0751, 289.0720, 169.0134, 125.0239, 96.9633
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer^{7H}	25.8	seeds, wine	$C_{67}H_{54}O_{28}$	1305.2791	1305.2723	652.1325*	5.2	1305.2727, 1153.2628, 863.1900, 729.1432, 577.1323, 575.1185, 407.0766, 289.0716, 169.0145, 125.0239*, 96.9630
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer^{8H}	26.5	seeds, wine	$C_{67}H_{54}O_{28}$	1305.2714	1305.2723	652.1321*	-0.7	1305.2749, 1154.2715, 1025.1906, 727.1361, 575.1181, 407.0753, 289.0716*, 169.0146, 125.0244
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer^{9H}	27.4	seeds, wine	$C_{67}H_{54}O_{28}$	1305.2723	1305.2723	652.1326*	-3.1	1305.2616, 1153.2689, 1017.2089, 865.2050, 729.1430, 575.1162, 407.0763, 289.0719*, 169.0128, 125.0244, 96.9600
(epi)cat/(epi)cat/(epi)catgallate /(epi)catgallate/(epi)catgallate pentamer^{1H}	29.2	seeds	$C_{96}H_{74}O_{42}$	-	1814.3807	948.1770*	-	1441.2988, 1169.2572, 949.1656. 863.1782, 729.1482, 575.1175, 407.0763, 289.0728*, 169.0130, 125.0232
(epi)cat/(epi)cat/(epi)catgallate /(epi)catgallate/(epi)catgallate pentamer^{2H}	29.9	seeds	$C_{96}H_{74}O_{42}$	-	1814.3807	948.6802*	-	1688.1245, 1594.3152, 1441.3203, 1153.2534, 863.1858, 575.1198, 577.1371, 407.0772, 289.0717*, 169.0145, 125.0245, 96.9631
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate/(epi)catgallate pentamer^{1H}	28.3	seeds	$C_{89}H_{70}O_{38}$	-	1690.3647	872.1680*	-	1745.2960, 1443.3120, 1153.2562, 1017.2147, 863.1788, 729.1490, 577.1273, 575.1161, 407.0761, 289.0717, 169.0140, 125.0243*, 96.9610
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate/(epi)catgallate pentamer^{2H}	29.1	seeds	$C_{89}H_{70}O_{38}$	-	1690.3647	872.1702*	-	1593.3043, 1441.3135, 1305.2498, 1153.2810, 1017.2271, 863.1783, 729.1472, 575.1179, 407.0760, 289.0720*, 169.0132, 125.0241, 96.9602
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate/(epi)catgallate pentamer^{3H}	29.5	seeds	$C_{89}H_{70}O_{38}$	-	1690.3647	872.1707*	-	1746.2891, 1154.2490, 1015.2018, 872.6732, 727.1367, 575.1211, 407.0765, 289.0719, 169.0143, 125.0244*
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate/(epi)catgallate pentamer^{4H}	30.4	seeds	$C_{89}H_{70}O_{38}$	-	1690.3647	872.1725*	-	1595.3312, 1441.2972, 1153.2614, 1017.1989, 863.1844, 575.1186, 289.0683*, 169.0157, 125.0243, 96.9629

(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate/(epi)catgallate pentamer[5H]	30.8	seeds	$C_{89}H_{70}O_{38}$	-	1690.3647	872.1702*	-	1593.3134, 1305.2512, 1153.2805, 1016.1945, 863.1747, 729.1380, 577.1319, 575.1202, 407.0765, 289.0715*, 169.0140, 125.0238, 96.9623
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)catgallate pentamer[1H]	27.7	seeds	$C_{82}H_{66}O_{34}$	-	1566.3486	796.1647*	-	1593.3190, 1441.3043, 1151.2291, 1017.2133, 865.1899, 729.1417, 575.1196, 407.0750, 289.0715, 169.0135, 125.0252*, 96.9599
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)catgallate pentamer[2H]	28.8	seeds	$C_{82}H_{66}O_{35}$	-	1566.3486	796.1642*	-	1594.3145, 1441.2839, 1151.2235, 863.1825, 575.1232, 407.0744, 289.0700, 169.0138, 125.0244*
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)catgallate pentamer[3H]	29.5	seeds	$C_{82}H_{66}O_{36}$	-	1566.3486	796.1650*	-	-
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)catgallate pentamer[4H]	30.4	seeds	$C_{82}H_{66}O_{37}$	-	1566.3486	796.1654*	-	1441.2955, 1016.2052, 863.1868, 577.1329, 575.1190, 407.0761, 289.0682*, 169.0159, 125.0246, 96.9634
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)catgallate pentamer[5H]	30.8	seeds, wine	$C_{82}H_{66}O_{38}$	-	1566.3486	796.1645*	-	1016.1917, 863.1734, 727.1309, 577.1304, 575.1200, 407.0769, 289.0717*, 169.0138, 125.0234, 96.9638
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)catgallate hexamer[1H]	31.6	seeds	$C_{97}H_{78}O_{40}$	-	1854.412	940.6957*	-	1151.2208, 1016.2008, 863.1637, 729.1410, 575.1240, 407.0776, 289.0693*, 169.0142, 125.0245, 96.9635
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)catgallate hexamer[2H]	32.2	seeds	$C_{97}H_{78}O_{40}$	-	1854.412	940.1930*	-	1151.2241, 1017.2047, 865.1860, 729.1455, 575.1178, 577.1312, 407.0765, 289.0711, 287.0567*, 169.0142, 125.0238, 96.9625
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)catgallate hexamer[3H]	32.4	seeds	$C_{97}H_{78}O_{40}$	-	1854.412	940.7002*	-	1151.2512, 1017.1826, 863.1941, 730.1478, 575.1152, 407.0711, 287.0558*, 125.0242
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)catgallate hexamer[4H]	32.8	seeds	$C_{97}H_{78}O_{40}$	-	1854.412	940.1926*	-	1151.2449, 1016.2036, 863.1790, 729.1443, 577.1349, 575.1187, 407.0776, 289.0717, 287.0557*, 169.0138, 125.0242, 96.9602
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)catgallate hexamer[5H]	33.4	seeds	$C_{97}H_{78}O_{40}$	-	1854.412	940.1952*	-	1303.2681, 1152.2423, 1016.2042, 863.1792, 729.1400, 577.1301, 575.1205, 407.0759, 289.0721*, 169.0155, 125.0235, 96.9604
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)catgallate	34.6	seeds	$C_{97}H_{78}O_{40}$	-	1854.412	940.6983*	-	1304.2615, 1151.2421, 1017.1979, 863.1858, 577.1322, 575.1186,

hexamer[6H]									407.0768, 289.0707, 287.0558, 169.0145, 125.0246*, 96.9613
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)catgallate hexamer[7H]	35.2	seeds	$C_{97}H_{78}O_{40}$	-	1854.412	940.6989*		-	1304.2601, 1152.7552, 1016.2021, 863.1772, 729.1484, 577.1348, 575.1183, 407.0756, 287.0552*, 169.0144, 125.0244, 96.9593
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat/ (epi)catgallate heptamer[1H]	35.6	seeds	$C_{112}H_{90}O_{46}$	-	2142.4754	1084.7313 *	722.8203	-	-
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat/ (epi)catgallate heptamer[2H]	36.8	seeds	$C_{112}H_{90}O_{46}$	-	2142.4754	1084.7292 *	722.8192	-	-
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/(epi)cat/ (epi)catgallate heptamer[3H]	37.9	seeds	$C_{112}H_{90}O_{46}$	-	2142.4754	1084.7335 *	722.822	-	-
epigallocat gallate[H]	14.4	seeds, wine	$C_{22}H_{18}O_{11}$	457.0783	457.0771	-	-	2.6	457.0735, 289.0722*, 137.0428, 125.0247
gallocat gallate[H]	15.8	seeds	$C_{22}H_{18}O_{11}$	457.0778	457.0771	-	-	1.5	457.0685, 289.0726, 137.0226, 125.0255*

[a] Exact sequence of the monomeric units in isomeric compounds is not known. Abbreviations: (epi)cat: catechin or epicatechin, (epi)gallocat: gallocatechin or epigallocatechin, (epi)catgallate: catechin gallate or epicatechin gallate, theor: theoretical/calculate molar mass, exp: experimental molar mass. Superscripts indicate isomer number, while [H] denotes HILIC.

Table S4: Summary of ions detected by RPLC-ESI-MS analysis of condensed tannins in wine and seed extracts. Base peak ions are indicated with an asterisk.

Compound[a]	t_R (min)	samples	molecular formula	$[M - H]^-$ exp	$[M - H]^-$ theor	$[M - 2H]^{2-}$	Δ ppm	MS^E fragments
monomer^{1RP} (catechin)	9.59	seeds, wine	$C_{15}H_{14}O_6$	289.0716	289.0712	-	1.4	289.0715*, 245.0826, 137.0243, 109.0305
monomer^{2RP} (epicatechin)	13.3	seeds, wine	$C_{15}H_{14}O_6$	289.072	289.0712	-	2.8	289.0717*, 245.0826, 137.0249, 109.0295
(epi)cat/(epi)cat dimer^{1RP}	9.14	seeds, wine	$C_{30}H_{26}O_{12}$	577.1334	577.1346	-	-2.1	577.1331, 425.0867, 407.0756, 289.0718*, 248.0816, 137.0245, 125.0256, 96.9603
(epi)cat/(epi)cat dimer^{2RP}	9.32	seeds, wine	$C_{30}H_{26}O_{12}$	577.1339	577.1346		-1.2	577.1335, 425.0852, 407.0758*, 289.0722, 245.0814, 137.0234, 125.0245, 96.9610
(epi)cat/(epi)cat dimer^{3RP}	11.8	seeds, wine	$C_{30}H_{26}O_{12}$	577.1332	577.1346		-2.4	577.1346, 425.0880, 407.0772, 289.0727*, 245.0806, 137.0219, 125.0243, 96.9604
(epi)cat/(epi)cat dimer^{4RP}	12.8	seeds, wine	$C_{30}H_{26}O_{12}$	577.1334	577.1346		-2.1	577.1329, 425,0862, 407.0761*, 289.0715, 245.0819, 125.0251
(epi)cat/(epi)cat dimer^{5RP}	13.3	seeds	$C_{30}H_{26}O_{12}$	577.132	577.1346		-4.5	577.1360, 424.9877, 407.0770, 289.0717*, 245.0824, 137.0250, 125.0245, 96.9606
(epi)cat/(epi)cat dimer^{6RP}	13.8	seeds	$C_{30}H_{26}O_{12}$	577.1339	577.1346		-1.2	577.1340, 425.0906, 407.0771, 289.0718*, 245.0845, 137.0237, 125.0267, 96.9613
(epi)cat/(epi)cat dimer^{7RP}	14.3	seeds	$C_{30}H_{26}O_{12}$	577.1349	577.1346		0.5	577.1370, 425.0797, 407.0738, 289.0712*, 125.0437, 137.0254, 125.0250, 96.9620
(epi)cat/(epi)cat dimer^{8RP}	15.5	seeds	$C_{30}H_{26}O_{12}$	577.135	577.1346		0.7	577.1259, 425.0875, 407.0760, 289.0665*, 137.0193, 125.0268, 96.9618
(epi)cat/(epi)cat dimer^{9RP}	17.0	seeds, wine	$C_{30}H_{26}O_{12}$	577.136	577.1346		2.4	577.1384, 425.0852, 407.0782, 289.0730*, 245.0686, 137.0248, 125.0257, 96.9586
(epi)cat/(epi)cat dimer^{10RP}	21.5	seeds, wine	$C_{30}H_{26}O_{12}$	577.1346	577.1346		0	577.1344, 425.0854, 407.0773, 289.0731*, 245.0466, 137.0253, 125.0247, 96.9598
(epi)cat/(epi)cat dimer^{11RP}	24.7	seeds	$C_{30}H_{26}O_{12}$	577.134	577.1346		-1	577.1379, 425.0984, 289.0739, 125.0248*, 96.9573
(epi)cat/(epi)cat/(epi)cat trimer^{1RP}	4.41	seeds	$C_{45}H_{38}O_{18}$	865.1954	865.198	-	-3	865.1991*, 713.1577, 577.1257, 575.1260, 407.0710, 289.0691, 125.0236
(epi)cat/(epi)cat/(epi)cat trimer^{2RP}	4.68	seeds, wine	$C_{45}H_{38}O_{18}$	865.1984	865.198	-	0.5	865.2000, 713.1465, 575.1229, 289.0726, 125.0251*, 96.9623
(epi)cat/(epi)cat/(epi)cat trimer^{3RP}	10.5	seeds	$C_{45}H_{38}O_{18}$	865.1956	865.198	-	-2.8	865.2061, 712.1247, 577.1333, 423.0761, 407.0757, 289.0714*, 245.0464, 125.0257, 96.9619
(epi)cat/(epi)cat/(epi)cat trimer^{4RP}	11.4	seeds, wine	$C_{45}H_{38}O_{18}$	865.197	865.198		-1.2	865.2002*, 713.1400, 577.1287, 407.0777, 289.0706, 245.0476, 169.0181, 125.0253, 96.9602
(epi)cat/(epi)cat/(epi)cat trimer^{5RP}	12.2	seeds, wine	$C_{45}H_{38}O_{18}$	865.1964	865.198		-1.8	865.1952, 577.1346, 407.0728, 289.0715*, 169.0122, 125.0235, 96.9595
(epi)cat/(epi)cat/(epi)cat trimer^{6RP}	12.6	seeds, wine	$C_{45}H_{38}O_{18}$	865.1955	865.198		-2.9	865.1977, 575.1242, 407.0781*, 289.0703, 245.0412, 125.0248, 96.9604
(epi)cat/(epi)cat/(epi)cat trimer^{7RP}	13.0	seeds, wine	$C_{45}H_{38}O_{18}$	865.1968	865.198		-1.4	865.1954, 714.1545, 577.1351, 407.0736, 289.0702*, 245.0419, 125.0250, 96.9637

(epi)cat/(epi)cat/(epi)cat trimer^{8RP}	13.5	seeds	$C_{45}H_{38}O_{18}$	865.1963	865.198		-1.8	865.1927, 575.1192, 407.0807, 289.0703*, 245.0806, 125.0238, 96.9607
(epi)cat/(epi)cat/(epi)cat trimer^{9RP}	15.7	seeds, wine	$C_{45}H_{38}O_{18}$	865.1957	865.198		-2.7	865.1845, 575.1211, 425.0881, 407.0720, 289.0719, 125.0248*, 96.9610
(epi)cat/(epi)cat/(epi)cat trimer^{10RP}	15.9	seeds, wine	$C_{45}H_{38}O_{18}$	865.1963	865.198		-1.8	865.1944, 577.1375, 425.0904, 407.0779*, 289.0750, 169.0126, 125.0258, 96.9605
(epi)cat/(epi)cat/(epi)cat trimer^{11RP}	16.3	seeds, wine	$C_{45}H_{38}O_{18}$	865.1984	865.198		0.5	865.1968, 713.1564, 695.1420, 577.1320, 425.0875, 407.0786, 289.0711*, 287.0562, 245.0469, 125.0256
(epi)cat/(epi)cat/(epi)cat trimer^{12RP}	18.1	seeds, wine	$C_{45}H_{38}O_{18}$	865.2017	865.198		4.3	865.1934, 577.1405, 575.1221, 423.0752, 407.0790*, 287.0562, 169.0154, 125.0247, 96.9599
(epi)cat/(epi)cat/(epi)cat trimer^{13RP}	21.5	seeds	$C_{45}H_{38}O_{18}$	865.1974	865.198		-0.7	865.1988, 575.1214, 407.0792, 289.0725*, 245.0468, 125.0252, 96.9591
(epi)cat/(epi)cat/(epi)cat/(epi)cat tetramer^{1RP}	9.84	seeds	$C_{60}H_{50}O_{24}$	1153.2515	1153.2614	576.1247*	-8.6	1153.2448, 577.1203, 407.0706, 289.0728, 245.0813*, 125.0262, 96.9628
(epi)cat/(epi)cat/(epi)cat/(epi)cat tetramer^{2RP}	12.0	seeds, wine	$C_{60}H_{50}O_{24}$	1153.2557	1153.2614	576.1257*	-4.9	1153.2670, 865.1706, 577.1308, 425.0887, 407.0760, 289.0721*, 245.0821, 125.0239, 96.9613
(epi)cat/(epi)cat/(epi)cat/(epi)cat tetramer^{3RP}	14.4	seeds	$C_{60}H_{50}O_{24}$	1153.2567	1153.2614	576.1260*	-4.1	1153.2540, 863.1701, 577.1417, 407.0786, 289.0721*, 245.0439, 169.0138, 125.0254, 96.9607
(epi)cat/(epi)cat/(epi)cat/(epi)cat tetramer^{4RP}	17.5	seeds, wine	$C_{60}H_{50}O_{24}$	1153.2598	1153.2614	576.1264*	-1.4	1153.2618, 866.1978, 575.1191, 449.0851, 407.0757, 289.0708*, 287.0567, 243.0299, 161.0238, 125.0252
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat pentamer^{1RP}	14.3	seeds	$C_{75}H_{62}O_{30}$	-	1441.3248	720.1576*	-	1442.2886, 1153.2611, 1017.1985, 865.1854, 407.0744, 289.0717*, 245.0441, 125.0252, 96.9622
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat pentamer^{2RP}	17.4	seeds	$C_{75}H_{62}O_{30}$	1441.2987	1441.3248	720.1573*	-18.1	-
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat pentamer^{3RP}	18.1	seeds	$C_{75}H_{62}O_{30}$	-	1441.3248	720.1573*	-	1441.3137, 1153.2720, 1017.2078, 865.2009, 575.1255, 407.0806, 289.0471*, 245.0409, 125.0251, 96.9595
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat pentamer^{4RP}	19.0	seeds, wine	$C_{75}H_{62}O_{30}$	1441.3076	1441.3248	720.1565*	-11.9	1441.3158, 1153,2769, 863.1718, 711,1321, 575.1214, 407.0766, 289.0732*, 287.0576, 245.0458, 125,0247
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat pentamer^{5RP}	21.2	seeds	$C_{75}H_{62}O_{30}$	-	1441.3248	720.1602*	-	-
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat hexamer^{1RP}	19.6	seeds	$C_{90}H_{74}O_{36}$	-	1730.529	864.6902*	-	1154.2360, 1017.1873, 865.1885, 575.1147, 407.0773. 287.0567*, 243.0309, 125.0258, 96.9586
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat hexamer^{2RP}	20.2	seeds	$C_{90}H_{74}O_{36}$	-	1730.529	864.1885*	-	1441.2859, 1153.2809, 863.1817, 709.1213, 575.1172, 407.0772, 289.0726*, 287.0556, 169.0157, 125.0243
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat heptamer^{1RP}	20.3	seeds	$C_{105}H_{86}O_{42}$	-	2018.4594	1008.2135*	-	1153.2267, 863.2043, 577.1238, 575.1144, 407.0756, 287.0575*, 243.0311, 169.0140, 125.0250, 96.9598
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)cat heptamer^{2RP}	20.9	seeds	$C_{105}H_{86}O_{42}$	-	2018.4594	1008.7177*	-	1153.2325, 865.2114, 575.1184, 407.0758, 289.0705*, 287.0574, 125.0248

gallocat[RP]	5.17	wine	$C_{15}H_{14}O_7$	305.0665	305.0661	-	1.3	305.0669, 289.0720, 177.0188, 125.0247*
epigallocat[RP]	8.98	wine	$C_{15}H_{14}O_7$	305.0663	305.0661	-	0.7	305.0655, 289.0700, 177.0186, 125.0244*
(epi)gallocat dimer[1RP]	3.12	wine	$C_{30}H_{26}O_{14}$	609.1239	609.1244		-0.8	609.1281, 423.0714*, 305.0663, 177.0197, 125.0239
(epi)gallocat dimer[2RP]	4.99	wine	$C_{30}H_{26}O_{14}$	609.1232	609.1244		-2	609.1234, 423.0660, 177.0174, 125.0257*, 96.9604
(epi)cat/(epi)gallocat dimer[1RP]	5.18	wine	$C_{30}H_{26}O_{13}$	593.1282	593.1295		-2.2	593.1284, 425.0880, 407.0760, 305.0570, 289.0715, 177.0190, 125.0248*
(epi)cat/(epi)gallocat dimer[2RP]	6.17	wine	$C_{30}H_{26}O_{13}$	593.1278	593.1295		-2.9	-
(epi)cate/(epi)gallocat dimer[3RP]	7.67	wine	$C_{30}H_{26}O_{13}$	593.1286	593.1295		-1.5	593.1257, 425.0882, 407.0776, 289.0723, 245.0803, 177.0210*, 125.0247, 96.9605
(epi)cat/(epi)gallocat dimer[4RP]	9.82	wine	$C_{30}H_{26}O_{13}$	593.1298	593.1295		0.5	593.1268, 423.0731, 407.0780, 289.0717, 243.0297, 177.0201, 125.0245, 96.9623
(epi)cat/(epi)gallocat/ (epi)gallocat trimer[1RP]	6.46	wine	$C_{45}H_{38}O_{20}$	897.1854	897.1878		-2.7	897.1746, 289.0718*, 245.0845, 125.0247, 96.9642
(epi)cat/(epi)gallocat/ (epi)gallocat trimer[2RP]	7.88	wine	$C_{45}H_{38}O_{20}$	897.1902	897.1878		2.7	897.1836, 729.1721, 407.0734, 289.0721*
(epi)cat/(epi)gallocat/ (epi)gallocat trimer[3RP]	9.86	wine	$C_{45}H_{38}O_{20}$	897.1841	897.1878		-4.1	897.1713, 881.1874, 713.1463, 593.1303, 407.0778, 303.0510, 289.0728, 243.0313, 177.0179, 125.0244*, 96.9623
(epi)cat/(epi)cat/ (epi)gallocat trimer[1RP]	3.28	wine	$C_{45}H_{38}O_{19}$	881.1912	881.1929		-1.9	881.1927, 423.0717*, 305.0658, 177.0193, 125.0241
(epi)cat/(epi)cat/ (epi)gallocat trimer[2RP]	8.85	wine	$C_{45}H_{38}O_{19}$	881.1921	881.1929		-0.9	881.1856, 593.1176, 423.0659, 407.0746, 305.0650, 289.0711, 125.0251*
(epi)cat/(epi)cat/ (epi)gallocat trimer[3RP]	10.3	wine	$C_{45}H_{38}O_{19}$	881.1902	881.1929		-3.1	881.1908, 575.1086, 289.0712, 125.0241*, 96.9620
(epi)cat/(epi)cat/ (epi)gallocat trimer[4RP]	11.8	wine	$C_{45}H_{38}O_{19}$	881.1906	881.1929		-2.6	881.1932, 577.1335, 407.0756, 289.0714*, 177.0198, 125.0251, 96.9615
(epi)cat/(epi)cat/ (epi)gallocat trimer[5RP]	14.5	wine	$C_{45}H_{38}O_{19}$	881.1915	881.1929		-1.6	881.1947, 609.1073, 593.1446, 577.1279, 407.0757, 289.0732, 177.0203, 125.0245*, 96.9611
(epi)catgallate[1RP]	20.0	seeds, wine	$C_{22}H_{18}O_{10}$	441.0822	441.0822	-	0	441.0827, 289.0717, 169.0146*, 125.0247
(epi)catgallate[2RP]	21.2	seeds	$C_{22}H_{18}O_{10}$	441.0799	441.0822	-	-5.2	-
(epi)cat/(epi)catgallate dimer[1RP]	15.0	seeds, wine	$C_{37}H_{30}O_{16}$	729.1436	729.1456		-2.7	729.1718, 577.1385, 407.0711, 287.0567, 243.0295, 177.0195, 125.0244*, 96.9623
(epi)cat/(epi)catgallate dimer[2RP]	15.9	seeds	$C_{37}H_{30}O_{16}$	729.1468	729.1456		1.8	729.1522, 713.1584, 577.1331, 425.0867, 407.0779, 289.0739*, 125.0258, 96.9617
(epi)cat/(epi)catgallate dimer[3RP]	16.1	seeds	$C_{37}H_{30}O_{16}$	729.1445	729.1456		-1.5	729.1497, 577.1343, 407.0770*, 289.0724, 245.0423, 125.0255, 96.9603
(epi)cat/(epi)catgallate dimer[4RP]	17.3	seeds, wine	$C_{37}H_{30}O_{16}$	729.1448	729.1456		-1.1	729.1434, 577.1345, 559.0887, 441.0821, 407.0772*, 289.0727, 169.0158, 125.0251

(epi)cat/(epi)catgallate dimer[5RP]	26.7	seeds	$C_{37}H_{30}O_{16}$	729.1471	729.1456		2.1	729.1497, 577.1292, 575.1273, 407.0771*, 289.0720, 169.0158, 125.0247, 96.9609
(epi)catgallate/ (epi)catgallate dimer[1RP]	21.2	seeds	$C_{44}H_{34}O_{20}$	881.1577	881.1565		1.4	881.1548, 729.1445, 575.1183, 407.0763, 287.0558, 160.0146, 125.0252*
(epi)catgallate/ (epi)catgallate dimer[2RP]	29.0	seeds	$C_{44}H_{34}O_{20}$	881.1479	881.1565		-9.8	881.1532, 863.1848, 575.1227, 407.0739, 287.0562, 125.0242*, 96.9599
(epi)cat/(epi)catgallate/ (epi)catgallate trimer[1RP]	20.7	seeds	$C_{59}H_{46}O_{26}$	1169.2085	1169.2199	584.1085*	-9.8	1169.2224, 865.2104, 727.1367, 575.1167, 407.0773, 289.0718, 287.0572, 169.0150, 125.0254*
(epi)cat/(epi)cat/ (epi)catgallate trimer[1RP]	10.8	seeds	$C_{52}H_{42}O_{22}$	1017.2036	1017.2089	508.104*	-5.2	1017.1885, 865.2242, 407.0772, 289.0756*, 96.9606
(epi)cat/(epi)cat/ (epi)catgallate trimer[2RP]	13.8	seeds	$C_{52}H_{42}O_{22}$	1017.205	1017.2089	508.1002*	-3.8	1017.2150, 865.1918, 575.1224, 407.0762, 287.0564*, 287.0564, 125.0280, 96.9607
(epi)cat/(epi)cat/ (epi)catgallate trimer[3RP]	14.1	seeds, wine	$C_{52}H_{42}O_{22}$	1017.2126	1017.2089	508.1022*	3.6	1017.2121*, 865.1899, 729.1334, 577.1356, 407.0704, 289.0723, 128.0239, 96.9594
(epi)cat/(epi)cat/ (epi)catgallate trimer[4RP]	15.4	seeds	$C_{52}H_{42}O_{22}$	1017.2076	1017.2089	508.1034*	-1.3	1017.2195, 865.1843, 577.1317, 425.0910, 407.0789, 289.0707, 243.0355, 125.0254, 96.9611
(epi)cat/(epi)cat/ (epi)catgallate trimer[5RP]	16.3	seeds	$C_{52}H_{42}O_{22}$	1017.2078	1017.2089	508.1051*	-1.1	1017.2011, 865.1967*, 713.1526, 577.1321, 425.0882, 407.0783, 289.0718, 125.0256, 96.9603
(epi)cat/(epi)cat/ (epi)catgallate trimer[6RP]	18.2	seeds	$C_{52}H_{42}O_{22}$	1017.2067	1017.2089	508.1023*	-2.2	1017.2158, 865.1938, 729.1408, 577.1376, 575.1204, 407.0793, 289.0733*, 245.0550, 125.0250, 96.9611
(epi)cat/(epi)cat/ (epi)catgallate trimer[7RP]	21.1	seeds	$C_{52}H_{42}O_{22}$	1017.2048*	1017.2089	508.1*	-4	1017.2159, 881.1536, 865.2098, 729.1504, 575.1136, 407.0756, 287.0561*, 245.0429, 169.0124, 125.0251, 96.9609
(epi)cat/(epi)cat/ (epi)catgallate trimer[8RP]	22.1	seeds	$C_{52}H_{42}O_{22}$	1017.2072	1017.2089	508.0998*	-1.7	1017.2075, 865.1746, 729.1404, 575.1204, 407.0774*, 289.0710, 287.0574, 169.0158, 125.0251
(epi)cat/(epi)cat/ (epi)catgallate trimer[9RP]	25.4	seeds	$C_{52}H_{42}O_{22}$	1017.204	1017.2089	508.1006*	-4.8	1017.1902, 863.1974, 729.1422, 575.1202, 407.0786, 287.0586*, 169.0157, 125.0250, 96.9608
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer[1RP]	12.3	seeds	$C_{67}H_{54}O_{28}$	1305.2782	1305.2723	652.1302*	4.5	1305.2506, 1154.2625, 865.1965, 575.1149, 407.0748, 289.0712*, 245.0480, 125.0241, 96.9608
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer[1RP]	16.4	seeds, wine	$C_{67}H_{54}O_{28}$	1305.2625	1305.2723	652.1344*	-7.8	1305.2579, 1153.2380, 865.1965, 713.1517, 577.1323, 407.0780, 289.0719*, 245.0461, 177.0202, 125.0254, 96.9599
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer[1RP]	17.0	seeds	$C_{67}H_{54}O_{28}$	1305.2639	1305.2723	652.1306*	-6.4	1305.2614, 1154.2284, 1017.2135, 865.1997, 577.1390, 425.0859, 407.0786, 289.0732, 125.0260, 96.9590
(epi)cat/(epi)cat/(epi)cat /(epi)catgallate tetramer[1RP]	17.9	seeds	$C_{67}H_{54}O_{28}$	1305.2631	1305.2723	652.1321*	-7	1305.2648, 1153.2838, 1017.2156, 865.1938, 729.1469, 577.1400, 407.0791, 287.0560*, 245.0549, 125.0249, 96.9603
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer[1RP]	20.5	seeds	$C_{67}H_{54}O_{28}$	1305.2772	1305.2723	652.1298*	3.8	1305.2592, 1153.2607, 1017.2091, 865.1990, 577.1309, 407.0773, 303.0495, 287.0569, 245.0433, 125.0258*, 96.9617
(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer[1RP]	23.0	seeds	$C_{67}H_{54}O_{28}$	1305.2778	1305.2723	652.1309*	4.2	1305.2673, 863.1594, 729.1307, 575.1176, 407.0732, 289.0735, 169.0143*, 125.0240

(epi)cat/(epi)cat/(epi)cat/ (epi)catgallate tetramer^{1RP}	29.0	seeds	$C_{67}H_{54}O_{28}$	-	1305.2723	652.1299*	-0.7	1305.2465, 1151.2520, 1015.1899, 881.1568, 863.1841, 727.1315, 575.1180, 407.0744, 287.0554*, 243.0287, 169.0139, 125.0246, 96.9602
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)catgallate pentamer^{1RP}	19.2	seeds	$C_{82}H_{66}O_{35}$	-	1457.3197	796.1640*	-	1441.3102, 1153.2804, 1017.1918, 863.1782, 711.1333, 575.1194, 407.0763, 289.0723*, 243.0312, 169.0149, 125.0250, 96.9595
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)catgallate pentamer^{2RP}	22.7	seeds	$C_{82}H_{66}O_{35}$	-	1457.3197	796.1598*	-	1441.3328, 1303.2428, 1017.2066, 863.1827, 729.1434, 575.1175, 407.0722, 303.0532, 287.0567, 169.0147, 125.0247, 96.9589
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)catgallate pentamer^{3RP}	24.0	seeds	$C_{82}H_{66}O_{35}$	-	1457.3197	796.1618*	-	1153.2627, 1016.2014, 863.1757, 575.1132, 407.0765, 287.0571, 169.0157, 125.0256*
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/ (epi)catgallate hexamer^{1RP}	18.9	seeds	$C_{97}H_{78}O_{40}$	-	1746.3909	940.6921*	-	-
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/ (epi)catgallate hexamer^{2RP}	19.6	seeds	$C_{97}H_{78}O_{40}$	-	1746.3909	940.1945*	-	-
(epi)cat/(epi)cat/(epi)cat/ (epi)cat/(epi)cat/ (epi)catgallate hexamer^{3RP}	25.0	seeds	$C_{97}H_{78}O_{40}$	-	1746.3909	940.1943*	-	-
gallocatgallate^{1RP}	4.15	seeds	$C_{22}H_{18}O_{11}$	457.0795	457.0771	-	5.3	457.0653, 407.0894, 287.0542, 96.9602*
epigallocatgallate^{1RP}	14.5	seeds, wine	$C_{22}H_{18}O_{11}$	457.0774	457.0771	-	0.7	407.0803, 289.0723, 125.0241*, 96.9607

[a] Exact sequence of the monomeric units in isomeric compounds is not known. Abbreviations: (epi)cat: catechin or epicatechin, (epi)gallocat: gallocatechin or epigallocatechin, (epi)catgallate: catechin gallate or epicatechin gallate, theor: theoretical/calculated molar mass, exp: experimental molar mass. Superscripts indicate isomer number, while ^{H} denotes HILIC.

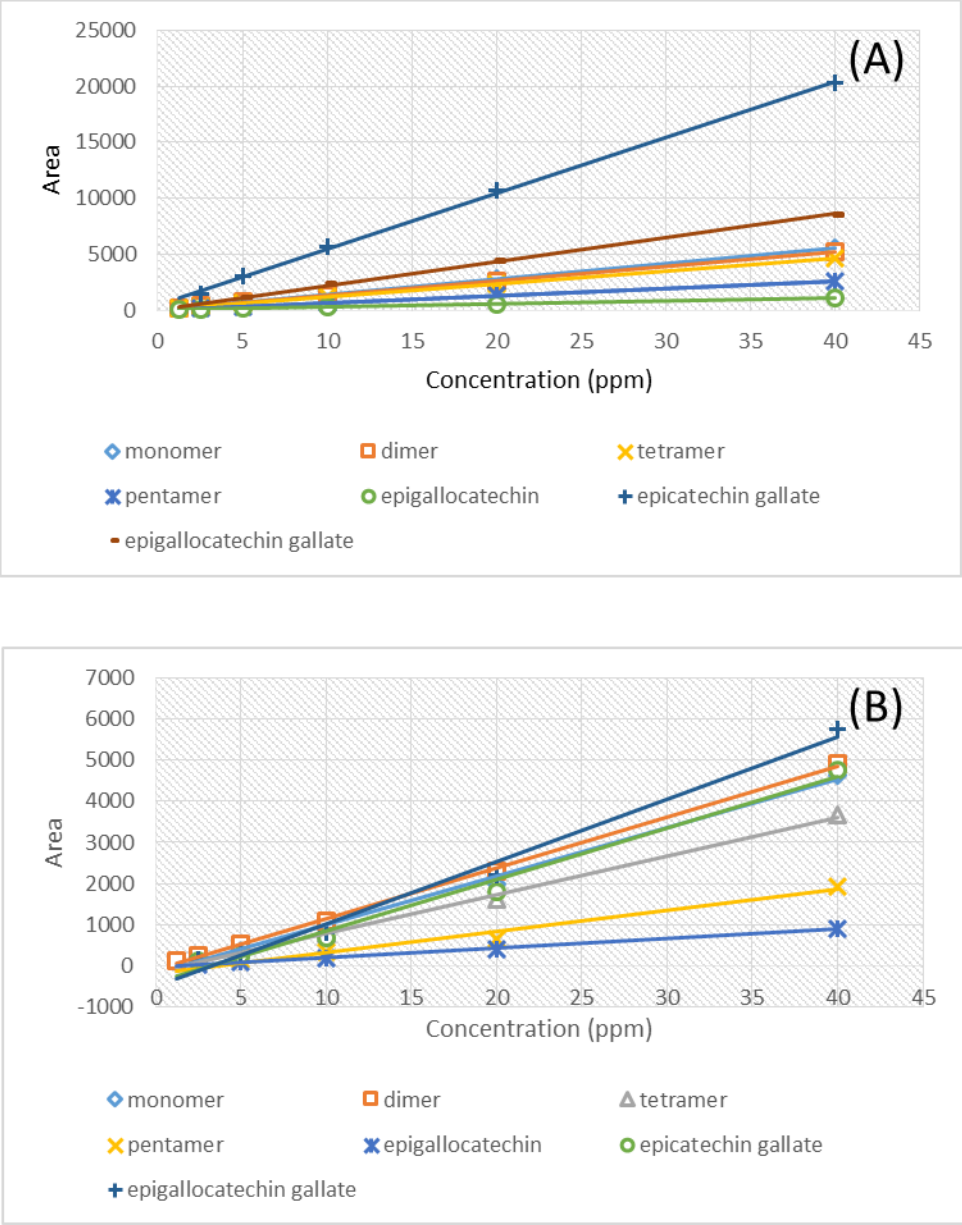

Figure S3: Calibration curves for PAC standards in (A) HILIC-UV (280 nm) and (B) RPLC-UV (280 nm). Injection volume: 10 µL.

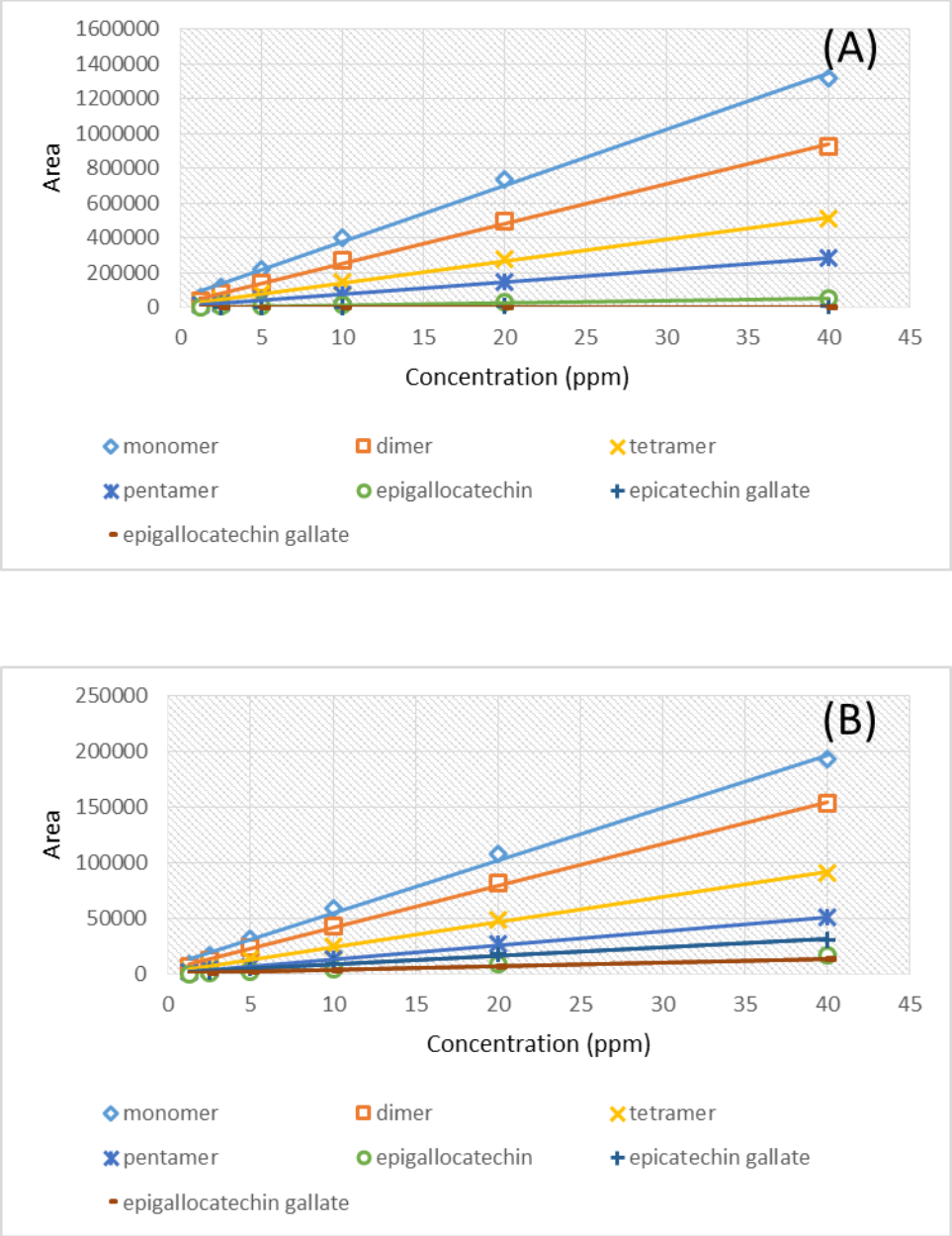

Figure S4: Calibration curves for PAC standards obtained by (A) HILIC-FLD (λ_{exc} 230 nm, λ_{em} 320 nm) and (B) HILIC-FLD (λ_{exc} 230 nm, λ_{em} 360 nm). Injection volume: 10 µL.

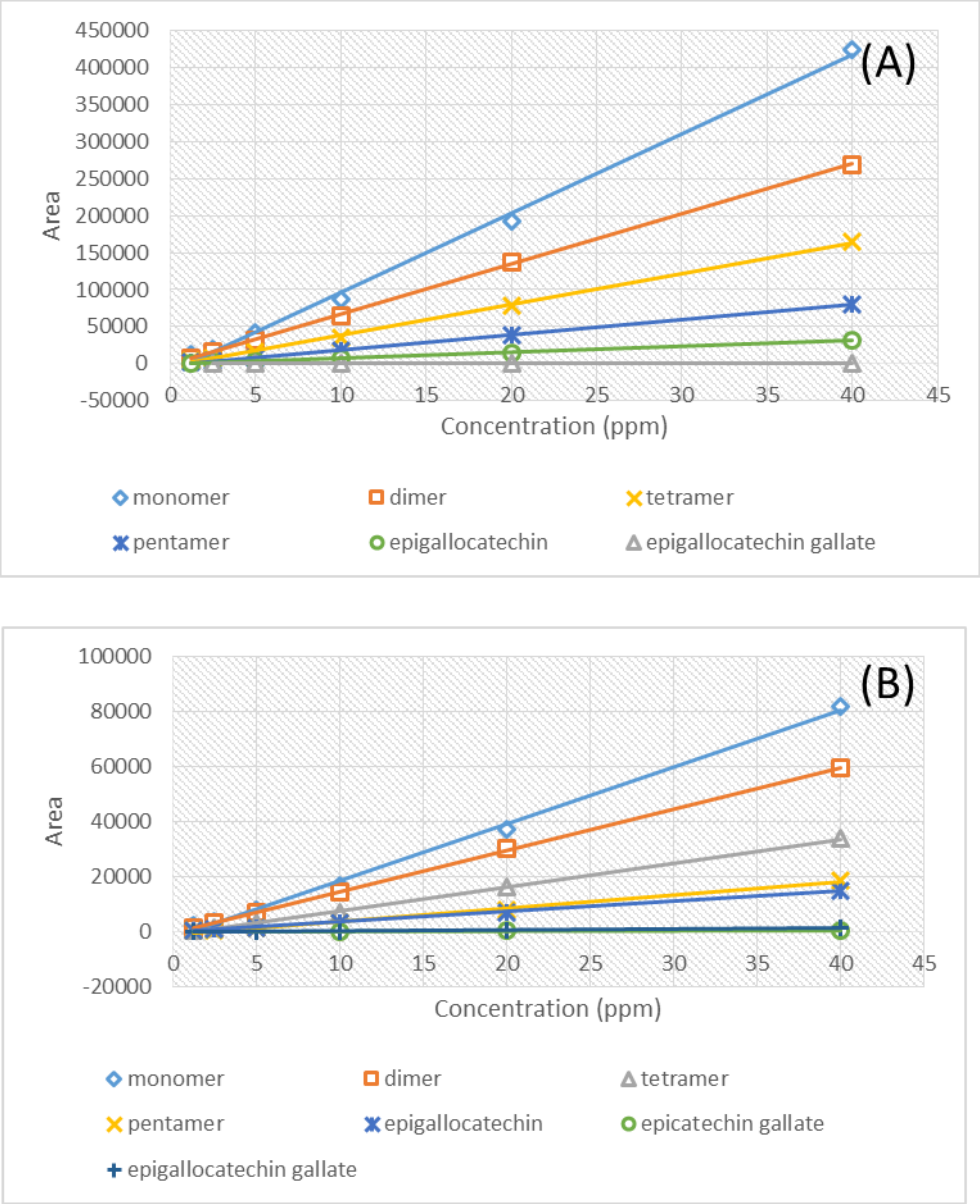

Figure S5: Calibration curves for PAC standards obtained by (A) RPLC-FLD (λ_{exc} 230 nm, λ_{em} 320 nm) and (B) RPLC-FLD (λ_{exc} 230 nm, λ_{em} 360 nm). Injection volume: 10 µL. Epicatechin gallate is not showed in (A) as it showed no response at these wavelengths.

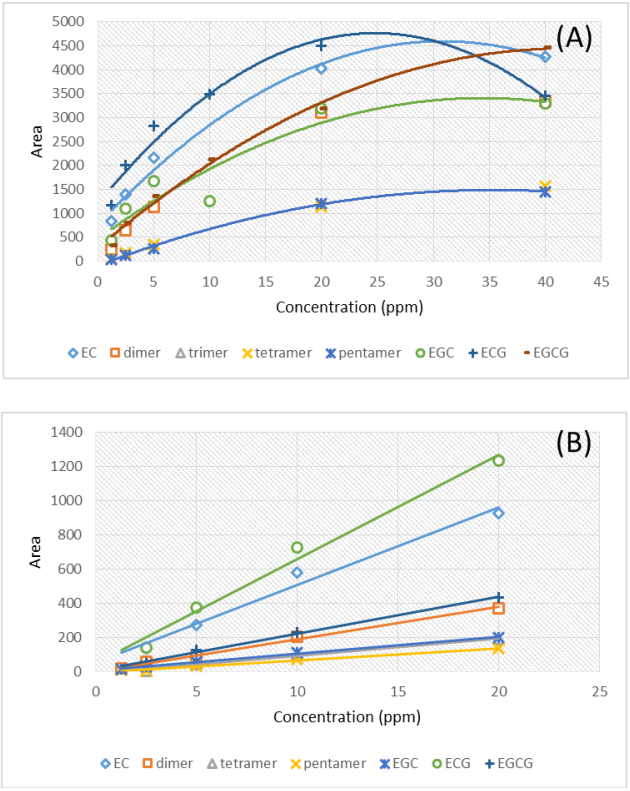

Figure S6: (A) Base peak ion calibration curves of PAC standards obtained by HILIC-MS full loop injection (10 µL). Demonstrates the saturation that occurs in HILIC-MS calibration. (B) Shows the base peak ion calibration curves of PAC standards obtained by HILIC-MS with reduced injection volume of 1.5 µL.

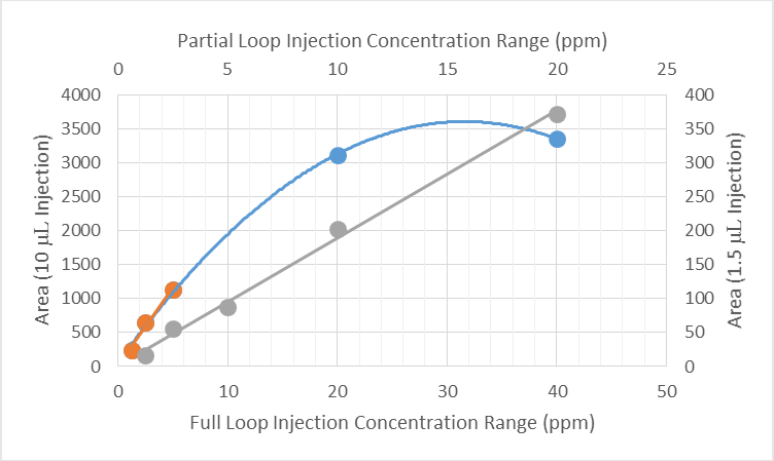

Figure S6C: A composite calibration curve for a PC dimer, obtained by HILIC-MS, showing the saturation of the MS with the full loop injection (blue line), the small linear range of the full loop injection (orange line) as well as the greater linear range with the partial loop injection (grey line).

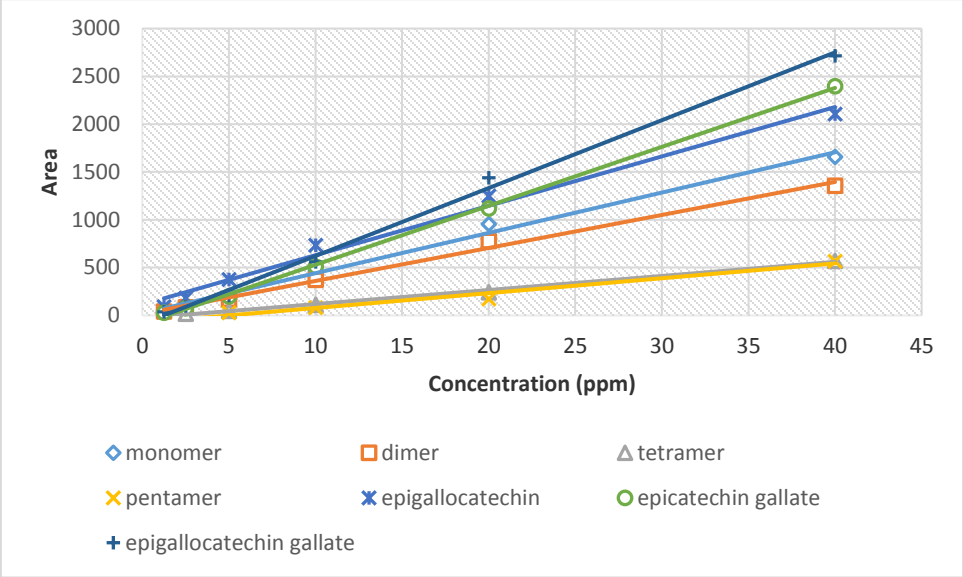

Figure S7: Base peak ion calibration curves of PAC standards obtained by RPLC-MS. Injection volume: 3 µL.

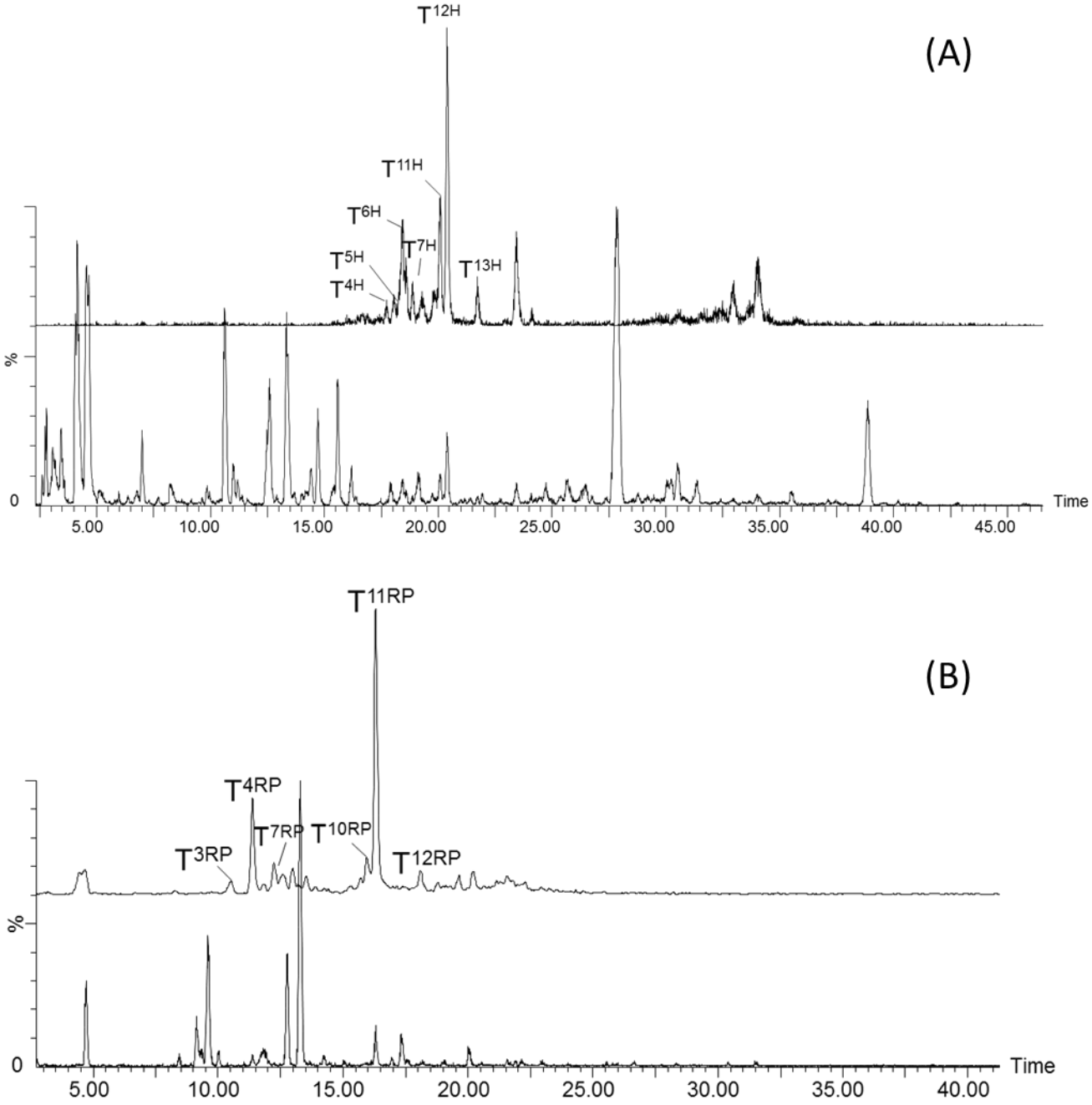

Figure S5: (A) HILIC base peak ion total ion chromatogram (bottom) and extracted ion chromatogram (865.1 m/z) (top) for a PC trimer. (B) Shows the same chromatograms in RP-LC.

Table S*3*: Quantitative data for PACs in a seed extract (ppm) obtained by HILIC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1H}	185	181	231[d]
monomer^{2H}			
monomer total	185	181	231
dimer^{1H}	4.93[a]	2.26	3.97
dimer^{2H}	3.06[a]	1.24	2.34
dimer^{3H}	5.86	1.06	6.51
dimer^{4H}	60.9[a]	20.0	12.9[c]
dimer^{5H}	n.q.	n.q.	1.05
dimer^{6H}	74.6	66.0	75.4[b]
dimer^{7H}	n.q.	n.q.	13.0
dimer^{8H}	5.14a	n.q.	1.08
dimer^{9H}	3.15[a]	n.d.	1.18
dimer total	158	90.5	117
trimer^{1H}	n.d.	n.q.	0.13
trimer^{2H}	0.36	0.55	0.45
trimer^{3H}	n.d.	0.35	0.28
trimer^{4H}	3.86	0.71	0.63
trimer^{5H}	3.69	1.29	1.17
trimer^{6H}	11.5	6.00	6.65
trimer^{7H}	3.92	2.66	1.70
trimer^{8H}	2.86	1.84	1.58
trimer^{9H}	6.81	3.51	1.89
trimer^{10H}	n.q.	5.70	1.76
trimer^{11H}	14.4	12.4	6.21
trimer^{12H}	34.1	26.2	13.7
trimer^{13H}	2.74	1.98	1.84
trimer^{14H}	10.3	4.92	5.24
trimer total	94.5	68.1	43.2
tetramer^{1H}	n.d.	n.d.	n.d.

tetramer[2H]	n.q.	1.50	n.q.
tetramer[3H]	7.32	3.29	1.99
tetramer[4H]	n.d.	n.d.	0.00
tetramer[5H]	13.28	2.30	3.92
tetramer[6H]	28.6	22.9	23.3[b]
tetramer total	49.2	30.0	29.2
pentamer[1H]	1.29	n.q.	n.d.
pentamer[2H]	4.89	2.55	n.d.
pentamer[3H]	n.d.	3.90	n.d.
pentamer[4H]	20.0	25.5	n.q.[c]
pentamer total	26.2	31.9	0.00
hexamer[1H]	1.59	1.24	1.43
hexamer[2H]	2.96	3.10	3.37
hexamer[3H]	7.23	7.89	8.82
hexamer total	11.8	12.2	13.6
heptamer[1H]	n.q.	3.21	n.q.
heptamer[2H]	5.65	11.4	9.22
heptamer total	5.65	14.7	9.22
octamer[1H]	n.d.	n.d.	n.q.
octamer[2H]	n.d.	7.63	n.q.
octamer total	n.d.	7.63	n.q.
nonamer[1H]	n.d.	n.d.	n.q.
nonamer total	n.d.	n.d.	n.q.
decamer[1H]	n.d.	n.d.	n.q.
decamer total	n.d.	n.d.	n.q.
(epi)gallocatechin[1H]	n.d.	n.d.	n.q.
(epi)gallocatechin total	n.d.	n.d.	n.q.
(epi)catechin gallate[1H]	n.d.	n.d.	2.82
(epi)catechin gallate[2H]	n.d.	n.d.	10.4
(epi)catechin gallate total	n.d.	n.d.	13.2
(epi)cat/(epi)catgallate dimer[3H]	16.3	n.d.	11.7

(epi)cat/(epi)catgallate dimer[5H]	5.98	n.d.	3.54
(epi)catgallate/(epi)catgallate dimer[1H]	4.01	n.d.	0.52
(epi)catgallate/(epi)catgallate dimer[2H]	2.51	n.d.	0.91
(epi)catgallate dimers total	28.8	n.d.	16.6
(epi)gallocatechin gallate[1H]	n.q.	n.q.	n.q.
(epi)gallocatechin gallate total	n.q.	n.q.	n.q.
Total procyanidins	530	436	443
Total prodelphinidins	n.q.	n.q.	n.q.
Total gallates	34.6	n.d.	29.9
TOTALS	**565**	**436**	**472**

[a] - overestimation due to co-elution

[b] - injection volume adapted to fit into calibration range for MS

[c] - analyte out of linear range of MS but not detected at lower injection volume therefore original value used

[d] - analyte out of linear range even at lowest injection volume

n.q.[e] - detected but no calibration curve for compound with specific detector

Table S*4*: Quantitative data for PACs in a seed extract (ppm) obtained by RPLC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1RP}	53.3	57.9	49.9
monomer^{2RP}	127	119	132[b]
monomer total	180	177	182
dimer^{1RP}	19.1[a]	14.4	19.9
dimer^{2RP}	15.5[a]	5.85	9.54
dimer^{3RP}	7.60[a]	21.9	18.6
dimer^{4RP}	42.5	46.1	44.3
dimer^{5RP}	5.48	n.d.	1.88
dimer^{6RP}	8.80[a]	2.51	3.06
dimer^{7RP}	3.95	n.d.	1.18
dimer^{8RP}	6.44	4.56	4.65
dimer^{9RP}	7.03	6.04	3.39
dimer^{10RP}	n.d.	n.d.	1.41
dimer total	116	101	106
trimer^{1RP}	n.d.	n.d.	3.16
trimer^{2RP}	14.6	14.6	15.7
trimer^{3RP}	4.55	3.51	5.49
trimer^{4RP}	n.d.	n.d.	3.55
trimer^{5RP}	n.d.	n.d.	3.81
trimer^{6RP}	n.d.	n.d.	2.77
trimer^{7RP}	13.2[a]	5.05	4.84
trimer^{8RP}	53.1	37.7	40.7
trimer^{9RP}	n.q.[b]	n.q.[b]	3.55
trimer total	85.4	60.9	83.5
tetramer^{1RP}	6.10	3.88	6.38
tetramer^{2RP}	20.0	17.5	23.3
tetramer total	26.1	21.4	29.7
pentamer^{1RP}	40.0[a]	18.3	15.2

pentamer[2RP]	30.2	27.3	22.4
pentamer total	70.2	45.6	37.7
epigallocatechin[1RP]	n.d.	n.d.	1.57
epigallocatechin total	0.00	0.00	1.57
epicatechin gallate[1RP]	n.d.	n.d.	18.4
epicatechin gallate total	0.00	0.00	18.4
(epi)cat/(epi)catgallate dimer[4RP]	15.5	n.d.	2.22
(epi)catgallate/(epi)catgallate dimer[1RP]	3.44	n.d.	2.47
(epi)catechin gallate dimers total	18.9	0.00	4.68
epigallocatechin gallate[1RP]	n.d.	n.d.	5.84
epigallocatechin gallate total	0.00	0.00	5.84
Total procyanidins	479	406	439
Total prodelphinidins	0.00	0.00	1.57
Total gallates	44.8	0.00	28.9
TOTALS	**523**	**406**	**470**

[a] - overestimation due to co-elution

[b] - injection volume adapted to fit into calibration range for MS

n.q.[b] - co-elution

Table S5: Quantitative data for PACs in Wine A (ppm) obtained by HILIC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1H}	n.d.	4.06[a]	0.60
monomer^{2H}	47.1[a]	33.5	29.3[b]
monomer total	47.1	37.5	29.9
dimer^{1H}	5.25[a]	0.04	0.37
dimer^{3H}	7.36[a]	3.79	3.14[b]
dimer^{5H}	22.2	20.4	16.6[b]
dimer total	34.8	24.3	20.1
trimer^{5H}	6.48[a]	1.41	0.78
trimer^{6H}	n.d.	0.45	n.q.
trimer^{8H}	2.20	0.36	n.q.
trimer^{9H}	0.30	n.q.	n.q.
trimer^{10H}	1.69	2.16	0.94
trimer^{11H}	5.03	3.48	1.10
trimer^{13H}	1.29	0.65	n.q.
trimer^{14H}	5.74	3.81	1.33
trimer total	22.7	12.3	4.16
tetramer^{1H}	n.d.	n.d.	n.q.
tetramer^{2H}	n.d.	n.d.	n.q.
tetramer^{3H}	n.d.	0.52	n.q.
tetramer^{4H}	n.d.	0.37	n.q.
tetramer^{5H}	n.d.	1.32	1.20
tetramer^{6H}	n.d.	n.d.	n.q.
tetramer total	0.00	2.20	1.20
pentamer^{2H}	n.d.	n.d.	n.q.
pentamer^{3H}	n.d.	0.63	n.q.
pentamer total	0.00	0.63	0.00
(epi)gallocatechin^{1H}	n.d.	n.d.	n.q.
(epi)gallocatechin^{2H}	n.d.	n.d.	0.48
(epi)gallocatechin total	0.00	0.00	0.48
(epi)cat/(epi)gallocat dimer^{1H}	n.d.	0.25	0.40
(epi)cat/(epi)gallocat dimer^{2H}	n.d.	0.11	0.07
(epi)cat/(epi)gallocat dimer^{3H}	3.73	0.24	1.17
(epi)cat/(epi)gallocat dimer^{4H}	n.d.	0.87	3.43
(epi)cat/(epi)gallocat dimer^{5H}	n.d.	0.66	3.45
(epi)cat/(epi)gallocat dimer^{6H}	1.33	1.40	7.45
(epi)gallocatechin dimer^{1H}	n.d.	n.d.	0.40
(epi)gallocatechin dimer^{2H}	n.d.	n.d.	1.71
(epi)gallocatechin dimer^{3H}	n.d.	n.d.	8.75
(epi)gallocatechin dimer total	5.07	3.52	26.8
(epi)cat/(epi)cat/(epi)gallocat trimer^{1H}	0.40	0.14	0.03

(epi)cat/(epi)cat/(epi)gallocat trimer^{2H}	n.d.	0.14	0.24
(epi)cat/(epi)cat/(epi)gallocat trimer^{3H}	1.58	0.18	0.15
(epi)cat/(epi)cat/(epi)gallocat trimer^{4H}	0.67	n.d.	0.22
(epi)cat/(epi)cat/(epi)gallocat trimer^{5H}	3.47	0.13	0.10
(epi)cat/(epi)cat/(epi)gallocat trimer^{6H}	3.19	0.90	1.20
(epi)cat/(epi)cat/(epi)gallocat trimer^{7H}	1.38	0.21	0.67
(epi)cat/(epi)cat/(epi)gallocat trimer^{8H}	n.d.	0.13	0.12
(epi)cat/(epi)cat/(epi)gallocat trimer^{9H}	n.d.	n.d.	0.30
(epi)gallocatechin trimer total	10.69	1.82	3.02
(epi)cat/(epi)cat/(epi)gallocat/(epi)gallocat tetramer^{1H}	n.d.	0.11	0.07
(epi)gallocatechin tetramer total	n.d.	0.11	0.07
(epi)cat/(epi)cat/(epi)gallocat/(epi)gallocat/(epi)gallocat pentamer^{1H}	n.d.	n.d.	n.d.
(epi)cat/(epi)cat/(epi)gallocat/(epi)gallocat/(epi)gallocat pentamer^{2H}	n.d.	n.d.	n.d.
(epi)cat/(epi)cat/(epi)gallocat/(epi)gallocat/(epi)gallocat pentamer^{3H}	n.d.	0.05	0.21
(epi)cat/(epi)cat/(epi)gallocat/(epi)gallocat/(epi)gallocat pentamer^{4H}	n.d.	0.03	0.13
(epi)cat/(epi)cat/(epi)cat/(epi)gallocat/(epi)gallocat pentamer^{1H}	n.d.	0.03	0.07
(epi)cat/(epi)cat/(epi)cat/(epi)gallocat/(epi)gallocat pentamer^{2H}	n.d.	0.06	0.09
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)gallocat pentamer^{1H}	n.d.	0.07	0.16
(epi)cat/(epi)cat/(epi)cat/(epi)cat/(epi)gallocat pentamer^{2H}	n.d.	0.02	n.d.
(epi)gallocatechin pentamer total	0.00	0.18	0.50
(epi)gallocatechin gallate total	0.00	0.00	0.00
Total procyanidins	105	77.0	35.05
Total prodelphinidins	15.8	5.62	30.90
Total gallates	0.00	0.00	0.00
TOTALS	**120**	**82.6**	**66.0**

[a] - overestimation due to co-elution

[b] - injection volume adapted to fit into calibration range for MS

Table S6: Quantitative data for PACs in Wine A (ppm) obtained by RPLC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1RP}	26.7[a]	24.3[a]	15.0
monomer^{2RP}	11.2	10.3	9.63
monomer total	37.9	34.6	24.6
dimer^{1RP}	9.97	8.14	9.68
dimer^{2RP}	1.65	1.31	1.75
dimer^{3RP}	4.32[a]	2.48	1.90
dimer^{4RP}	11.4	8.33	5.69
dimer^{8RP}	4.19[a]	0.99	0.90
dimer total	31.6	21.3	19.9
trimer^{2RP}	4.07	5.54	2.16
trimer^{3RP}	n.d.	n.d.	1.12
trimer^{4RP}	n.d.	n.d.	0.76
trimer^{5RP}	0.91	n.d.	0.81
trimer^{7RP}	n.d.	1.07	0.74
trimer^{8RP}	n.d.	4.67	3.22
trimer total	4.98	11.28	8.81
(epi)gallocatechin^{1RP}	17.2[a]	15.7[a]	3.49
(epi)gallocatechin^{2RP}	n.d.	n.d.	1.29
(epi)gallocatechin total	17.2	15.7	4.78
(epi)gallocatechin dimer^{1RP}	n.d.	n.d.	0.89
(epi)catechin/(epi)gallocatechin dimer^{1RP}	n.d.	0.59	1.57
(epi)catechin/(epi)gallocatechin dimer^{2RP}	n.d.	0.60	0.21
(epi)catechin/(epi)gallocatechin dimer^{3RP}	n.d.	0.48	0.87
(epi)catechin/(epi)gallocatechin dimer^{4RP}	n.d.	n.d.	0.24
(epi)gallocatechin dimer total	0.00	1.67	3.79
(epi)cat/(epi)cat/(epi)gallocat trimer^{2RP}	0.00	0.25	0.18
(epi)cat/(epi)cat/(epi)gallocat trimer^{3RP}	0.00	0.26	0.13
(epi)cat/(epi)cat/(epi)gallocat trimer^{4RP}	0.00	0.70	0.09
(epi)cat/(epi)cat/(epi)gallocat trimer^{5RP}	0.00	0.20	0.15
(epi)gallocatechin trimers total	0.00	1.41	0.55
(epi)catechin gallate^{1RP}	n.d.	n.q.[c]	1.44
(epi)catechin gallate total	0.00	0.00	1.44
(epi)cat/(epi)catgallate dimer^{4RP}	1.12	n.d.	0.06
(epi)catgallate dimer total	1.12	0.00	0.06
Total procyanidins	74.4	67.1	53.4
Total prodelphinidins	17.2	18.8	9.11
Total gallates	3.80	n.d.	1.50
TOTALS	95.4	85.9	64.0

[a] - overestimation due to co-elution

n.q.[c] - detected but no calibration curve for compound with specific detector

Table S7: Quantitative data for PACs in Wine B (ppm) obtained by HILIC-UV-FLD-MS.

	UV	FLD	MS
monomer[1H]	n.d.	2.28[a]	0.36
monomer[2H]	33.8	21.5	22.6[b]
monomer total	33.8	23.8	22.9
dimer[1H]	n.d.	n.d.	0.29
dimer[2H]	n.d.	n.d.	0.11
dimer[3H]	n.d.	2.38	1.49
dimer[5H]	18.7	17.6	19.0[b]
dimer total	18.7	20.0	20.9
trimer[5H]	16.2[a]	1.50	1.75
trimer[10H]	2.89	1.93	2.09[b]
trimer[11H]	7.87[a]	4.12	1.62
trimer[13H]	2.88[a]	0.28	0.47
trimer[14H]	5.78	5.20	4.64[b]
trimer total	35.6	13.0	10.6
tetramer[1H]	n.d.	n.d.	0.36
tetramer[2H]	n.d.	n.q.	0.28
tetramer[3H]	n.d.	0.18	0.26
tetramer[4H]	n.d.	n.q.	0.28
tetramer[5H]	n.d.	0.01	0.53
tetramer[6H]	n.d.	n.d.	0.43
tetramer total	0.00	0.19	2.14
(epi)gallocatechin[1H]	n.d.	n.d.	n.q.[a]
epigallocatechin[2H]	n.d.	n.d.	0.88
epigallocatechin total	0.00	0.00	0.96
(epi)gallocatechin dimer[2H]	n.d.	n.d.	0.46
(epi)gallocatechin dimer[3H]	n.d.	n.d.	1.84
(epi)gallocatechin dimer total	0.00	0.00	2.30
Total procyanidins	88.1	57.0	56.6
Total prodelphinidins	n.d.	n.d.	3.26
Total gallates	n.d.	n.d.	n.d.
TOTALS	**88.1**	**57.0**	**59.8**

[a] - overestimation due to co-elution

[b] - injection volume adapted to fit into calibration range for MS

Table S8: Wine B ppm values obtained in RPLC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1RP}	27.8[a]	23.6[a]	11.5
monomer^{2RP}	8.83	8.05	7.85
monomer total	36.6	31.7	19.4
dimer^{1RP}	18.8	9.79	12.4
dimer^{2RP}	n.d.	1.20	0.99
dimer^{4RP}	7.33	5.61	5.08
dimer total	26.1	16.6	19.2
trimer^{2RP}	5.56	0.00	1.93
trimer^{3RP}	n.d.	n.d.	0.63
trimer^{5RP}	n.d.	2.06	0.76
trimer^{8RP}	0.95	2.22	2.05
trimer total	6.52	4.28	5.37
(epi)gallocatechin^{1RP}	n.d.	n.d.	3.92
(epi)gallocatechin^{2RP}	n.d.	n.d.	1.39
(epi)gallocatechin total	0.00	0.00	5.31
(epi)gallocatechin dimer^{1RP}	ND	ND	3.73
(epi)gallocatechin dimer total	ND	ND	3.73
Total procyanidins	69.3	52.5	43.9
Total prodelphinidins	n.d.	n.d.	9.03
Total gallates	n.d.	n.d.	n.d.
TOTALS	**69.3**	**52.5**	**52.9**

[a] - overestimation due to co-elution

Table S9: Wine C ppm values obtained in HILIC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1H}	9.32	14.1	14.2
monomer total	9.32	14.1	14.2
dimer^{3H}	n.d.	1.28	1.76
dimer^{6H}	10.8	9.68	12.2
dimer total	10.8	11.	14.0
(epi)gallocatechin ^{1H}	n.d.	n.d.	7.69
(epi)gallocatechin ^{2H}	n.d.	n.d.	3.02
epigallocatechin total	0.00	0.00	10.7
(epi)gallocatechin dimer^{3H}	n.d.	n.d.	2.33
(epi)gallocatechin dimer total	0.00	0.00	2.33
Total procyanidins	20.1	25.0	28.2
Total prodelphinidins	n.d.	n.d.	13.0
Total gallates	n.d.	n.d.	n.d.
TOTALS	20.1	25.0	41.2

Table S10: Wine C ppm values obtained in RPLC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1RP}	19.5[a]	12.8[a]	7.04
monomer^{2RP}	7.89	6.76	6.08
monomer total	27.4	19.6	13.1
dimer^{1RP}	10.2	5.65	7.03
dimer^{3RP}	n.d.	0.54	0.46
dimer^{4RP}	n.d.	4.22	3.26
dimer^{8RP}	4.77[a]	0.44	0.51
dimer total	15.0	10.9	11.3
trimer^{2RP}	n.d.	n.d.	0.68
trimer^{8RP}	n.d.	1.67	0.94
trimer total	0.00	1.67	1.63
(epi)gallocatechin ^{1RP}	n.d.	n.d.	2.46
(epi)gallocatechin ^{2RP}	n.d.	n.d.	1.12
(epi)gallocatechin total	0.00	0.00	3.58
(epi)gallocatechin dimer^{1RP}	n.d.	n.d.	3.84
(epi)gallocatechin dimer total	0.00	0.00	3.84
Total procyanidins	42.4	32.1	26.0
Total prodelphinidins	n.d.	n.d.	3.58
Total gallates	n.d.	n.d.	n.d.
TOTALS	**42.4**	**32.1**	**29.6**

[a] - overestimation due to co-elution

Table S11: Wine D ppm values obtained in HILIC-UV-FLD-MS.

	UV	FLD	MS
monomer[1H]	32.9	55.4	58.8
monomer total	32.9	55.4	58.8
dimer[3H]	13.0	7.03	10.8
dimer[5H]	38.3	34.6	38.2
dimer total	51.3	41.6	49.0
trimer[10H]	n.d.	2.19	4.12
trimer[11H]	n.q.[c]	6.08	7.21
trimer[14H]	n.d.	6.31	7.98
trimer total	0.00	14.6	19.3
epigallocatechin[1H]	n.d.	n.d.	6.27
epigallocatechin[2H]	n.d.	n.d.	2.01
epigallocatechin total	0.00	0.00	8.28
Total procyanidins	84.2	112	127
Total prodelphinidins	n.d.	n.d.	8.28
Total gallates	n.d.	n.d.	n.d.
TOTALS	**84.2**	**112**	**135**

Table S12: Wine D ppm values obtained in RPLC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1RP}	38.9	34.2	26.6
monomer^{2RP}	28.9	26.1	30.4
monomer total	67.7	60.2	57.0
dimer^{1RP}	12.7	15.1	18.7
dimer^{4RP}	21.3[a]	41.8	13.3
dimer total	34.0	56.9	32.0
trimer^{2RP}	6.48	n.d.	4.67
trimer^{3RP}	n.d.	n.d.	1.74
trimer^{4RP}	n.d.	1.11	1.90
trimer^{5RP}	2.11	2.05	2.11
trimer^{7RP}	8.95[a]	1.70	1.05
trimer^{8RP}	28.0[a]	10.5	7.70
trimer^{9RP}	8.64[a]	1.05	0.86
trimer total	54.2	16.4	20.0
tetramer^{2RP}	n.d.	3.13	1.18
tetramer total	0.00	3.13	1.18
epigallocatechin^{1RP}	n.d.	n.d.	2.06
epigallocatechin^{2RP}	n.d.	n.d.	0.97
epigallocatechin total	0.00	0.00	3.03
Total procyanidins	156	137	110
Total prodelphinidins	n.d.	n.d.	3.03
Total gallates	n.d.	n.d.	n.d.
TOTALS	**156**	**137**	**113**

[a] - overestimation due to co-elution

Table S13: Wine E ppm values obtained in HILIC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1H}	13.1	26.4	29.0
monomer total	13.1	26.4	29.0
dimer^{3H}	6.84	0.00	3.89
dimer^{5H}	23.2	17.6	20.0
dimer total	30.1	17.6	23.9
trimer^{10H}	n.d.	1.57	1.55
trimer^{14H}	n.d.	6.61	1.29
trimer total	0.00	8.17	2.84
epigallocatechin^{1H}	n.d.	n.d.	12.0
epigallocatechin^{2H}	n.d.	n.d.	3.02
epigallocatechin total	n.d.	n.d.	15.0
Total procyanidins	43.1	52.1	55.7
Total prodelphinidins	n.d.	n.d.	15.0
Total gallates	n.d.	n.d.	n.d.
TOTALS	**43.1**	**52.1**	**70.6**

Table S14: Wine E ppm values obtained in RPLC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1RP}	27.0[a]	12.7	10.0
monomer^{2RP}	11.8	19.6	10.9
monomer total	38.8	32.4	21.0
dimer^{1RP}	9.63	9.13	5.87
dimer^{4RP}	9.61	20.1[a]	11.2
dimer total	19.2	29.2	17.0
trimer^{2RP}	10.0	5.35	1.49
trimer^{3RP}	n.d.	n.d.	0.81
trimer^{4RP}	n.d.	n.d.	0.63
trimer^{5RP}	1.02	0.78	0.84
trimer^{7RP}	n.d.	n.d.	0.37
trimer^{8RP}	40.0[a]	2.90	2.05
trimer total	51.1	9.03	6.20
epigallocatechin^{1RP}	n.d.	n.d.	2.84
epigallocatechin^{2RP}	n.d.	n.d.	1.10
epigallocatechin total	0.00	0.00	3.94
Total procyanidins	109	70.6	44.2
Total prodelphinidins	n.d.	n.d.	3.94
Total gallates	n.d.	n.d.	n.d.
TOTALS	**109**	**70.6**	**48.1**

[a] - overestimation due to co-elution

Table S15: Wine F ppm values obtained in HILIC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1H}	11.8[a]	10.5[a]	1.71
monomer total	11.8	10.5	1.71
dimer^{3H}	n.d.	n.d.	0.25
dimer^{5H}	8.98	8.06	9.19
dimer total	8.98	8.06	9.43
trimer^{10H}	n.d.	n.q.	0.12
trimer^{11H}	n.d.	0.17	0.08
trimer^{14H}	n.d.	0.40	0.61
trimer total	0.00	0.57	0.81
(epi)gallocatechin^{1H}	n.d.	n.d.	n.q.
(epi)gallocatechin^{2H}	n.d.	n.d.	n.q.
(epi)gallocatechin total	0.00	0.00	0.00
(epi)gallocatechin dimer^{2H}	n.d.	n.d.	0.93
(epi)gallocatechin dimer^{3H}	n.d.	n.d.	2.69
(epi)gallocatechin dimer total	0	0	3.62
Total procyanidins	20.8	19.1	12.0
Total prodelphinidins	n.d.	n.d.	3.62
Total gallates	n.d.	n.d.	n.d.
TOTALS	**20.8**	**19.1**	**15.6**

[a] - overestimation due to co-elution

Table S16: Wine F ppm values obtained in RPLC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1RP}	15.5[a]	7.29[a]	5.01
monomer^{2RP}	3.45	3.65	3.62
monomer total	18.9	10.9	8.63
dimer^{1RP}	5.15	5.04	5.56
dimer^{2RP}	n.d.	0.83	0.33
dimer^{4RP}	n.d.	1.54	1.28
dimer total	5.15	7.41	7.17
(epi)gallocatechin^{1RP}	n.d.	n.d.	1.52
(epi)gallocatechin^{2RP}	n.d.	n.d.	1.03
(epi)gallocatechin total	0.00	0.00	2.56
(epi)gallocatechin dimer^{1RP}	n.d.	n.d.	0.32
(epi)gallocatechin dimer total	0	0	0.32
Total procyanidins	24.1	18.4	15.8
Total prodelphinidins	n.d.	n.d.	2.88
Total gallates	n.d.	n.d.	n.d.
TOTALS	**24.1**	**18.4**	**18.7**

[a] - overestimation due to co-elution

Table S17: Wine G ppm values obtained in HILIC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1H}	16.7[a]	10.4[a]	1.27
monomer total	16.7	10.4	1.27
dimer^{1H}	n.d.	n.q.	n.q.
dimer^{3H}	n.d.	0.21	0.28
dimer^{5H}	11.5	8.90	10.2
dimer total	11.5	9.12	10.5
trimer^{10H}	n.d.	n.q.	n.q.
trimer^{11H}	n.d.	0.16	n.q.
trimer^{14H}	n.d.	0.61	0.64
trimer total	0.00	0.77	0.64
(epi)gallocatechin^{1H}	n.d.	n.d.	n.q.
(epi)gallocatechin^{2H}	n.d.	n.d.	n.q.
(epi)gallocatechin total	0.00	0.00	0.00
(epi)gallocatechin dimer^{2H}	n.d.	n.d.	0.97
(epi)gallocatechin dimer^{3H}	n.d.	n.d.	4.05
(epi)gallocatechin dimer total	0.00	0.00	5.02
Total procyanidins	28.2	20.2	12.4
Total prodelphinidins	n.d.	n.d.	5.02
Total gallates	n.d.	n.d.	n.d.
TOTALS	**28.2**	**20.2**	**17.4**

[a] - overestimation due to co-elution

Table S18: Wine G ppm values obtained in RPLC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1RP}	17.3[a]	8.85[a]	4.99
monomer^{2RP}	4.26	4.45	4.67
monomer total	21.6	13.3	9.67
dimer^{1RP}	4.18	5.86	6.48
dimer^{4RP}	n.d.	2.01	1.52
dimer total	4.18	7.87	8.00
(epi)gallocatechin ^{1RP}	n.d.	n.d.	1.67
(epi)gallocatechin ^{2RP}	n.d.	n.d.	0.94
epigallocatechin total	0.00	0.00	2.61
(epi)gallocatechin dimer^{1RP}	n.d.	n.d.	0.39
(epi)gallocatechin dimer total	0	0	0.39
Total procyanidins	25.8	21.2	17.7
Total prodelphinidins	n.d.	n.d.	3.00
Total gallates	n.d.	n.d.	n.d.
TOTALS	**25.8**	**21.2**	**20.7**

[a] - overestimation due to co-elution

Table S19: Wine H ppm values obtained in HILIC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1H}	12.4[a]	10.9[a]	1.31
monomer total	12.4	10.9	1.31
dimer^{3H}	n.d.	n.q.	0.32
dimer^{5H}	10.1	10.7	11.5[b]
dimer total	10.1	10.7	11.8
trimer^{10H}	n.d.	0.34	n.q.
trimer^{11H}	n.d.	0.42	n.q.
trimer^{14H}	2.01	0.81	1.35
trimer total	2.01	1.57	1.35
(epi)gallocatechin^{1H}	n.d.	n.d.	n.q.
(epi)gallocatechin^{2H}	n.d.	n.d.	n.q.
(epi)gallocatechin total	0.00	0.00	0.00
(epi)gallocatechin dimer^{2H}	n.d.	n.d.	2.01
(epi)gallocatechin dimer^{3H}	n.d.	n.d.	6.38
(epi)gallocatechin dimer total	0	0	8.39
Total procyanidins	24.5	23.2	14.5
Total prodelphinidins	n.d.	n.d.	8.39
Total gallates	n.d.	n.d.	n.d.
TOTALS	**24.5**	**23.2**	**22.9**

[a] - overestimation due to co-elution
[b] - injection volume adapted to fit into calibration range for MS

Table S20: Wine H ppm values obtained in RPLC-UV-FLD-MS.

	UV	FLD	MS
monomer^{1RP}	21.3[a]	13.1[a]	6.05
monomer^{2RP}	3.64	3.93	3.74
monomer total	24.9	17.0	9.79
dimer^{1RP}	6.36	7.29	8.31
dimer^{4RP}	n.d.	2.41	1.62
dimer total	6.36	9.71	9.93
trimer^{10RP}	n.d.	n.d.	0.50
trimer total	0.00	0.00	0.50
(epi)gallocatechin ^{1RP}	n.d.	n.d.	1.85
(epi)gallocatechin ^{2RP}	n.d.	n.d.	1.03
(epi)gallocatechin total	0.00	0.00	2.89
(epi)gallocatechin dimer^{1RP}	n.d.	n.d.	1.25
(epi)gallocatechin dimer total	0	0	1.25
Total procyanidins	31.3	26.7	20.2
Total prodelphinidins	n.d.	n.d.	4.14
Total gallates	n.d.	n.d.	n.d.
TOTALS	**31.3**	**26.7**	**23.1**

[a] - overestimation due to co-elution

Table S21: Wine I ppm values obtained in HILIC-UV-FLD-MS.

	UV	FLD	MS
monomer[1H]	16.1[a]	12.0[a]	1.42
monomer total	16.1	12.	1.42
dimer[3H]	n.d.	0.06	0.19
dimer[5H]	8.97	5.85	6.95[b]
dimer total	8.97	5.91	7.14
trimer[10H]	n.d.	n.q.	n.q.
trimer[11H]	n.d.	n.q.	n.q.
trimer[14H]	n.d.	0.05	n.q.
trimer total	0.00	0.05	0.00
epigallocatechin[1H]	n.d.	n.d.	n.q.
epigallocatechin[2H]	n.d.	n.d.	n.q.
epigallocatechin total	0.00	0.00	0.00
Total procyanidins	25.0	17.9	8.56
Total prodelphinidins	n.d.	n.d.	n.d.
Total gallates	n.d.	n.d.	n.d.
TOTALS	**25.0**	**17.9**	**8.56**

[a] - overestimation due to co-elution

[b] - injection volume adapted to fit into calibration range for MS

Table S22: Wine I ppm values obtained in RPLC-UV-FLD-MS.

	UV	FLD	MS
monomer[1RP]	20.5[a]	10.1[a]	6.43
monomer[2RP]	5.63	5.41	5.68
monomer total	26.1	15.5	12.1
dimer[1RP]	16.3[a]	4.65	5.16
dimer[4RP]	n.d.	1.18	1.24
dimer total	16.3	5.83	6.40
epigallocatechin[1RP]	n.d.	n.d.	1.89
epigallocatechin[2RP]	n.d.	n.d.	1.87
epigallocatechin total	0.00	0.00	3.77
Total procyanidins	42.4	21.4	22.3
Total prodelphinidins	n.d.	n.d.	3.77
Total gallates	n.d.	n.d.	n.d.
TOTALS	**42.4**	**21.4**	**26.0**

[a] - overestimation due to co-elution

Chapter 4

General conclusions and future recommendations

4.1. GENERAL CONCLUSIONS

Condensed tannins are important constituents of red wines. However, their structural complexity and diversity have precluded their complete characterisation and quantification to date. Bulk spectrophotometric methods may give information regarding the total quantity of these compounds, but for characterisation of individual compounds more powerful analytical tools such as high performance liquid chromatography (HPLC) are required.

Condensed tannins comprise three groups of compounds, namely procyanidins (flavan-3-ol oligomers), prodelphinidins (trihydroxylated flavan-3-ol oligomers) and gallated procyanidins (galloylated flavan-3-ol oligomers). Since each of the monomeric units of these condensed tannins contain two chiral centres, the structural diversity and complexity of these molecules increase exponentially with their degree of polymerisation.

Extensive research has focussed on this group of wine constituents in recent years, with several limitations being encountered. The first and potentially most detrimental hindrance in the quantification of condensed tannins has been the lack of commercially available standards for the higher molecular weight (MW) compounds. As a consequence, quantification is often performed in terms of (epi)catechin equivalents, with the assumption being made that the higher MW compounds would have the same response factors. To address this limitation, the first objective of the current study was to isolate suitable procyanidin (PC) standards of various degrees of polymerisation (DP) using semi-preparative HPLC, and to use these standards to determine the relative response factors (RRFs) of these compounds compared to (epi)catechin. For this particular class of condensed tannin, the response factor in m/v units was found to decrease with increasing DP in ultraviolet (UV), fluorescence (FLD) and electrospray ionisation mass spectrometry (ESI-MS) detection modes. The data obtained for PCs of DP 1-5 were extrapolated to obtain estimated RRFs for higher MW compounds using these detectors.

Next, the effect of the mobile phase composition on the response factors for each of the classes of proanthocyanidins (PACs) was investigated. It was found that response factors remained relatively constant within the range of mobile phase composition used in hydrophilic interaction chromatography (HILIC). In contrast, response factors varied significantly within the range of mobile phase composition used in reversed phase liquid chromatography (RP-LC). However, since related compounds eluted within relatively small retention time windows, this effect was negligible and ultimately not taken into account in subsequent quantification.

A second major limitation in condensed tannin analysis is the fact that complete separation of this diverse family of compounds is currently not possible. The next objective of this study was therefore to develop and optimise both HILIC and RP-LC methods using three detectors (UV, FLD and MS) connected in series, and then to evaluate each of the methods and detectors for the detailed qualitative and quantitative analysis of condensed tannins in grapes and wine.

HILIC separation was performed on a 4.6 mm i.d. BEH Amide column, which had previously shown promise in the analysis of other flavonoids. With this method, compounds eluted in order of DP, and separation of isomers was also achieved within the respective elution windows of each DP compound. RP-LC separation was performed on a 2.1 mm i.d. superficially porous C18 column, which had shown superior separation performance to convention C18 columns. With this method, elution order was determined by the hydrophobicity of the compounds, with more polar compounds eluting first.

In terms of the detection modes compared, FLD was the most sensitive for PCs, with limits of detection (LODs) in the range of 0.003 – 5.99 ppm (mg/L). UV detection was the most suited for quantification of gallated PCs, which showed very low response factors in FLD. Neither of these detectors provided sufficient sensitivity to quantify prodelphinidins. ESI-MS proved to be critical for compound identification, as compounds first had to be identified before they could be quantified. Quantification was also done using ESI-MS, however this was more complicated than with the other two detectors. The complications arose due to the limited linear range in MS (as saturation of MS occurred at higher concentration levels), as well as the formation of multiple ionic species at the varying concentrations. For these reasons, two injection volumes were used in HILIC, where calibration in MS was performed at both injection levels in order to obtain values within the linear range of the MS. With this approach, good results were obtained and most detected compounds could be quantified using one or more of the detectors. Each of the three detectors proved important for the quantification of a particular class of PAC, and their hyphenation in series therefore greatly extends the application range of the developed methods.

Red wine samples as well as red grape seed extracts were analysed. Wine samples were prepared using a solid phase extraction method followed by roto-evaporation in order to concentrate the samples prior to analysis. A grape seed extract was prepared using an extraction solvent (2:1 acetone:water v/v), lyophilised and reconstituted in methanol. All samples were analysed using both HILIC- and RP-LC-UV-FLD-Q-TOF-MS methods, and quantified using calibration curves constructed using the standards and extended to higher DP compounds. 161 compounds were identified in HILIC-ESI-MS, where 90 of these were quantified. In RPLC-ESI-MS, 75 compounds were identified and 41 were quantified. PCs were detected and quantified in all samples, while gallated PCs were only detected in seeds and PDs in wine

samples. HILIC-ESI-MS was found to be more sensitive, most likely due to the use of an organic-rich mobile phase which is beneficial for ESI detection.

The seed sample was found to have the greatest tannin content of all the samples. While total tannin composition cannot be compared between different methods, the quantities for both wine and grape seed samples compared favourably with literature. UV overestimated the total PCs in samples due to its low selectivity. Overall, there was good agreement between the two separation modes, indicating the applicability of both methods for the quantification of condensed tannins in red grape and wine samples. There was also a relatively good agreement between the various detectors, with FLD and MS showing the best correlations with R^2 greater than 0.99 between HILIC and RP-LC.

The HILIC method was found to be more sensitive than RP-LC (FLD and MS, also UV), and provided equivalent separation performance. Based on these observations, HILIC-UV-FLD-Q-TOF-MS is proposed as the preferred method for the detailed qualitative and quantitative analysis of grape and wine condensed tannins.

The current study reports the most advanced method of its kind to date, and sheds new light into both the complexity and the possibilities as pertains to the analysis of PACs. The work presented therefore lays the foundation for further detailed investigation of these highly influential wine constituents.

4.2 FUTURE RECOMMENDATIONS

The lack of commercially available higher molecular weight standards is a major drawback in the study of PACs. In this study, reference compounds isolated from cocoa proved invaluable in determining relative response factors for PCs. It would be therefore be highly informative to isolate higher molecular weight PDs and gallated PCs from suitable natural product sources to determine accurate RRFs for these classes as a function of DP also. Based on the results reported here, grape seed extracts could be investigated as a source of gallated PCs. Access to such standards would allow to establish accurate RRF values for each of the classes of PACs found in wines and grapes. Although this requires extensive investment of time and cost, once established, these values can be used to quantify any molecule using calibration curves for cheap, commercially available standards in combination with the relevant RRFs.

For future chromatographic analyses of PACs, further validation of the HILIC-UV-FLD-Q-TOF-MS method would be recommended to demonstrate its suitability for routine quantification of real-life samples. Furthermore, the comprehensive combination of HILIC and RP-LC methods, which has been reported

before, can then be extended to quantitative analysis of PACs. The improved separation performance of HILIC×RP-LC would be highly beneficial for grape and wine analysis as a means of limiting co-elution.

Thus far the complexity of PACs has hampered their analysis, and the improved performance of the methods reported here may prove beneficial in a range of wine-related research fields. The methods could of course be used to study in further detail the chemical diversity of wine tannins in commercial wines. This method can also be used as reference method to investigate performance of bulk methods such as the methyl cellulose precipitable (MCP) tannin assay or the bovine-serum albumin (BSA) tannin assay. Finally, with ever improving analytical instrumentation, the methods reported here can be further improved for this application.